Role of Selected Candidate Genes In Autoimmune Pathogenesis of Vitiligo

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This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Prof. Rasheedunnisa Begum.**

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Place: Vadodara

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This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph. D. degree of The Maharaja Sayajirao University of Baroda by **Mr. Mitesh Kumar Dwivedi** is his original work. The entire research work and the thesis have been built up under my supervision.

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Dedicated to The Lord and

My Beloved Parents.....

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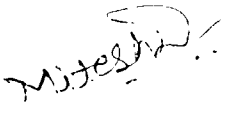

Mitesh Kumar Dwivedi

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ABBREVIATIONS

ACE	Angiotensin converting enzyme
AChE	Acetylcholine esterase
AIRE	Autoimmune regulatory region
AIS	Autoimmunity Susceptibility locus
APS	Ammonium persulfate
ASIP	Agouti signalling protein
ATChI	Acetlythiocholine iodide
BH_4	Tetrahydrobiopterin
bp	base pair (s)
bp	Base pairs
CAT	Catalase
CD	cluster of differentiation
CDNB	1 Chloro 2,4 dinitro benzene
CLEC11A	C-type Lectin Domain Family 11, Member A
COMT	Catechol O methyl transferase
CTLA 4	Cytotoxic T lymphocyte antigen 4
DAB	Diaminobenzidine
DDR1	Discoidin domain receptor tyrosine kinase 1
DOPA	dihydroxyphenylalanine
DTNB	5-5' Dithiobis (2-nitrobenzoic) acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-O, - O-bis (2 aminoethyl) N,N,N',N' tetra acetic acid
ESRI	Estrogen receptor gene 1

FOXD3	Forkhead box D3
G6PDH	Glucose 6 phosphate dehydrogenase
$G6PO_4$	Glucose 6 phosphate
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione S Transferase
GWAS	Genome-wide association studies
HEPES	N-2-hydroxyethly piperazine-N-2-ethane sulphonic acid
HGH	Human growth factor
HRP	Horse radish peroxidase
ICAM	Intercellular adhesion molucules
kb	kilobase (s)
KDa	Kilodalton
kDa	Kilodalton
KIT	tyrosine kinase receptor
LMP	Low molecular weight protein
LMP	Low molecular weight protein polypeptide
LPO	Lipid peroxidation
MC1R	Melanocortin-1 receptor
MCHR 1	melanin concentrating hormone receptor 1
MDA	Malondialdehyde
mM/M	Millimolar/Molar
MYG1	Melanocyte proliferating gene 1
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NBT	Nitro blue tetrazolium chloride monohydrate
ng	Nanogram
nm	Nanometer
NPY	Neuropeptide Y
NSV	Non-segmental vitiligo
OD	Optical density
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PMS	Phenazine methosulphate
PMSF	Phenylmethane sulphonyl fluoride
PVDF	Polyvinylidene fluoride
RFLP	Restriction fragment length polymorphism
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SLEV1	Vitiligo related systemic lupus erythematosus gene
SOD	Superoxide dismutase
SV	Segmental vitiligo
TAE	Tris acetate EDTA
TAP	Transporter associated with antigen processing protein
TAP	Transporter associated with antigen processing protein

TBA	Thio barbituric acid
TBE	Tris borate EDTA
TCA	Trichloroacetic acid
TEMED	N,N,N',N' Tetramethyl ethylenediamine
TGF-β	Transforming growth factor-β
TMB	Tetra methyl bezidine
TMP	Tetra methoxy propene
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl) aminomethane buffer
TRP-1	Tyrosinase-related protein-1
XO	Xanthine oxidase
µL/mL	Microlitres/millilitres

Note: The full forms of several rarely used abbreviations have been described within the text.

CHAPTER I

INTRODUCTION

"I look to a day when people will not be judged by the color of their skin, but by the content of their character."

Martin Luther King, Jr.

Vitiligo is an acquired, progressive, multifactorial, depigmenting disorder characterized by the appearance of circumscribed white macules in the skin due to chronic, progressive loss of functional melanocytes in the epidermis (Guerra *et al.*, 2010). The selective destruction of melanocytes results in the development of depigmented patches. The disease can have devastating consequences on an individual's relationships with others and internal feelings of self-worth. Immediate recognition of an individual's difference from the norm is through their appearance. Not only can appearance indicate an underlying difference but it is a source of deviance in itself as vitiligo is a cosmetically disfiguring condition. As such vitiligo is hardly a disease of medical significance but there is more of a social stigma attached to it because of cosmetic reasons. Although it might be viewed as minor disorder, these patches gradually increase in size and cause lot of psychological stress in the patient, self esteem and social interactions, particularly in patients with deeply pigmented skin (Kent *et al.*, 1996). Young women face more social stigma and suffer due to matrimonial problems.

Based on a few dermatological outpatient records the prevalence of vitiligo is found to be 0.5 to 1% of the world population (Taieb, 2007), 0.5 to 2.5 % in India (Handa *et al.*, 1999) and the states of Gujarat & Rajasthan have the highest prevalence i.e ~8.8% (Valia *et al.*, 1996). Vitiligo affects all with no predilection for gender or race, and usually starts in childhood or young adulthood. Manifestation begins before 20 years of age in 50% of the cases, while in 25% of the cases the onset is before 14 years of age (Kakourou, 2009). Familial cases of vitiligo are common indicating a hereditary factor; 6%-38% of vitiligo patients have family members with the disease (Ortonne *et al.*, 1983).

Though vitiligo has been extensively addressed in the past six decades, its etiology is still being debated (Taieb, 2000). Many possible causes of vitiligo have been

proposed, including stress, infections, mutations, neural factors, melatonin receptor dysfunction, and impaired melanocyte migration and/or proliferation. In addition, the accumulation of toxic intermediate products of melanin synthesis (Pawelek *et al.*, 1980; Moellmann *et al.*, 1982) the breakdown of free radical defense (Nordlund *et al.*, 1982) and the build-up of excessive quantities of hydrogen peroxide (Schallreuter *et al.*, 1991; Schallreuter *et al.*, 1994) have all been suggested to result in the self-destruction of pigment cells. An autoimmune etiology has also been proposed, and this is supported by the frequent association of vitiligo with autoimmune diseases (Ochi *et al.*, 1969; Macaron *et al.*, 1977) together with studies demonstrating that many vitiligo patients have autoantibodies and autoreactive T cells against melanocyte antigens (Naughton *et al.*, 1983; Bystryn *et al.*,1985). It is possible that these different causal factors can act independently or together to yield the same effect - namely, the disappearance of melanocytes from the skin (Le Poole *et al.*, 1993). Vitiligo is a complex, polygenic disorder (Strömberg *et al.*, 2008). Gene expression studies, allelic association studies of candidate genes and genome-wide linkage analyses are being used to discover new genes, and these studies have begun to shed light on the mechanisms of vitiligo pathogenesis (Spritz, 2008).

1.1 STRUCTURE OF SKIN

The skin is the largest body organ and functions as a metabolically active biological barrier separating internal homeostasis from the external environment. Depending on anatomic localization and environmental influences, the skin shows remarkable functional and structural diversity (Slominski and Wortsman, 2000). The dermal-epidermal junction is undulating in section; ridges of the epidermis, known as rete ridges, project into the dermis (Figure 1). The junction provides mechanical support for the epidermis and acts as a partial barrier against exchange of cells and large molecules. Below the dermis is a fatty layer, the panniculus adiposus, usually designated as 'subcutaneous'. This is separated from the rest of the body by a vestigial layer of striated muscle, the panniculus carnosus. The superficial epidermis is a stratified epithelium largely composed of keratinocytes that are formed by division of cells in the basal layer, and give rise to several distinguishable layers as they move outwards and progressively differentiate. Within the epidermis, there are

everal other cell populations, namely melanocytes, which donate pigment to the keratinocytes; Langerhans cells, which have immunological functions; and Merkel cells. The hair follicles comprise pockets of epithelium that are continuous with the superficial epidermis. They undergo intermittent activity throughout life. During the active phase, the follicle envelops at its base a small papilla of dermis. A bundle of smooth muscle fibres, the arrector pili, extends at an angle between the surface of the dermis and a point in the follicle wall. Above the insertion, the holocrine sebaceous gland opens by a short neck into the pilary canal, and some follicles in certain areas of the body, notably the axilla, have, in addition, an apocrine gland. Also derived from the epidermis and opening directly to the skin surface, are the eccrine sweat glands, present in every region of the body in densities of 100–600/cm^2. The basis of the dermis is a supporting matrix or ground substance in which polysaccharides and proteins are linked to produce macromolecules with a remarkable capacity for retaining water. Within and associated with this matrix are two kinds of protein fibres: collagen, which has great tensile strength and forms the major constituent of the dermis, and elastin, which makes up only a small proportion of the bulk. The cellular constituents of the dermis include fibroblasts, mast cells and histiocytes (monocytes/macrophages). The dermis has a very rich blood supply, although no vessels pass through the dermal-epidermal junction. The motor innervation of the skin is autonomic, and includes a cholinergic component to the eccrine sweat glands and adrenergic components to both the eccrine and apocrine glands, to the smooth muscle and the arterioles and to the arrector pili muscle. The sensory nerve endings are of several kinds: some are free, some terminate in hair follicles and others have expanded tips.

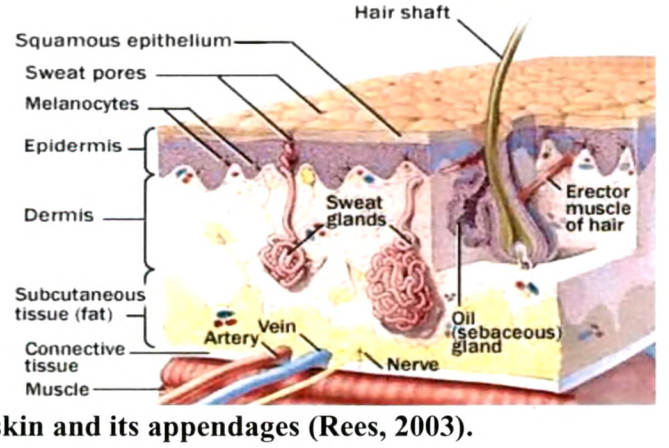

Figure 1: The skin and its appendages (Rees, 2003).

1.1.1 Colour of the skin:

Skin color is primarily due to the presence of a pigment called melanin. Both light and dark complexioned people have this pigment. Under normal conditions it is not the number of melanocytes in the skin that determine the degree of pigmentation but their level of activity. Skin color is also affected by red cells in blood flowing close to the skin. To a lesser extent, the color is affected by the fat under the skin and carotene a reddish-orange pigment in the skin.

1.1.2 Melanocytes:

The melanocytes are neural crest derived cells that migrate via mesenchyme into the epidermis and hair follicles during embryogenesis (Bolognia and Orlow, 2003). Apart form the skin, melanocytes are known to be present in other areas such as retinal pigment epithelium, uveal tract, inner ear and leptomeninges.

In the skin melanocytes reside in the basal layer of the epidermis and in the matrix of the hair follicle. They derive from the melanoblasts that originate from the neural crest from where they migrate during embryogenesis. Melanocytes are highly dendritic and these dendrites project into the malphigian layer of the epidermis where they transfer the melanosomes to keratinocytes (Jimbow *et al.,* 1999). Each epidermal melanocyte secretes melanosomes into approximately 36 keratinocytes in the neighborhood and this entire unit is called epidermal melanin unit (Figure 2).

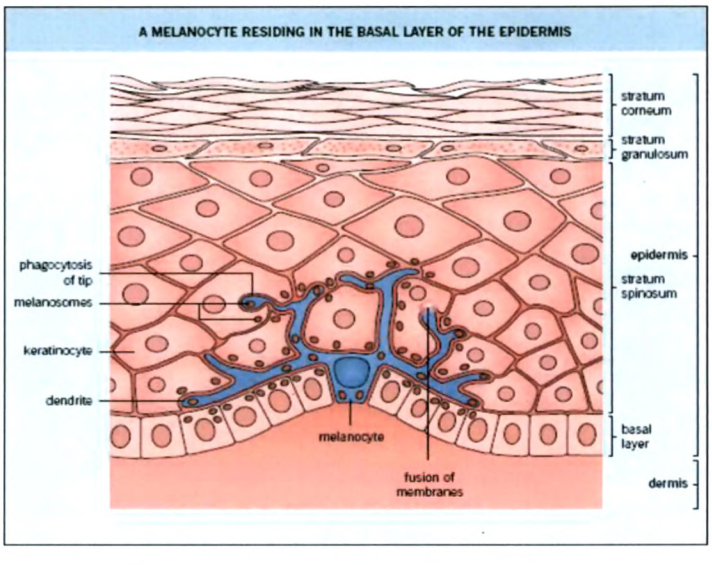

Figure 2. Epidermal melanin unit (Bolognia, Jorizzo, and Schaffer, *Dermatology*, 3rd edition).

Melanosomes are specialized subcellular organelles in which melanin is synthesized and deposited (Orlow, 1995). There are four stages in the maturation of melanosome:

Stage I, the "premelanosome" a spherical organelle with ill defined matrix filaments is seen;

Stage II, in which the typical elliptical shape of the melanosome is filled with a well defined filamentous or laminar matrix;

Stage III, with deposition of electron opaque melanin occurs on this matrix;

Stage IV, with complete opacification of melanosomal contents takes place by the melanin deposited therein (Orlow, 1995). The transition to stage II melanosomes involves elongation of the vesicle, and the appearance of distinct fibrillar structures. The production of internal matrix fibers and the maturation from stages I to II melanosomes depend on the presence of a structural protein termed Pmel 17 or gp100. A melanosomal protein called MART 1 forms a complex with Pmel 17 and thus plays an important role in melanogenesis by regulating the expression, stability, trafficking and processing of Pmel 17, which in turn regulates the maturation of melanosomes (Hoashi *et al.,* 2005).

1.1.3 Melanogenesis:

Pheomelanin and eumelanin are the two pigments formed during melanogenesis. Pheomelanin and eumelanin differ not only in colour but also in the size, shape and packaging of their granules (Slominski *et al.*, 2004). Both melanins derive from a common tyrosinase-dependent pathway with the same precursor, tyrosine (Figure 3).The obligatory step is hydroxylation of tyrosine to dopaquinone, from which L-DOPA can also be derived (Land *et al.*, 2000). From dopaquinone, the eumelanin and pheomelanin pathways diverge. Two enzymes crucial to eumelanogenesis are the tyrosinase-related proteins TRP1 (also known as GP75) and TRP2 (also known as dopachrome tautomerase). Pheomelanin is derived from conjugation by thiol-containing cysteine or glutathione. As a result, pheomelanin is more photolabile and can produce, among its by-products, hydrogen peroxide, superoxide and hydroxyl radicals, all known triggers of oxidative stress, which can cause DNA damage. Individual melanocytes typically synthesize both eumelanins and pheomelanins, with the ratio of the two being determined by a balance of variables, including pigment

enzyme expression and the availability of tyrosine and sulphydryl containing reducing agents in the cell.

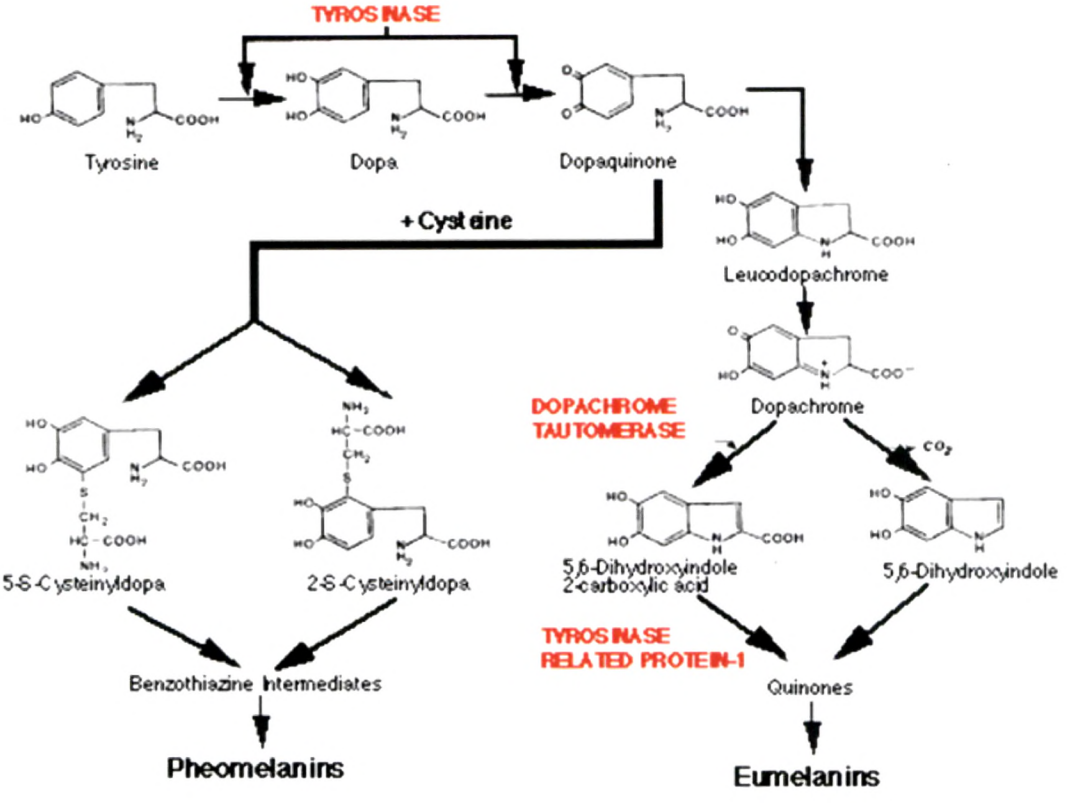

Figure 3. Melanin synthesis pathway.

Once melanin is produced, the melanosomes are transferred into the neighboring keratinocytes. The size of these organelles and their numbers are important in determining skin pigmentation. The melanosomes in black skin are larger than their counterparts in white skin and are packaged as single units rather than in groups. This has the effect of retarding their degradation in the keratinocytes and contributes to a higher level of skin pigmentation. It appears that association of melanosomes with microtubules and actin filaments via motor proteins, such as kinesin, dynein, and myosin V, is important for melanosome movement along the dendrites and for subsequent transfer to keratinocytes. Melanocyte dendricity and contact with keratinocytes is likely to be essential for the transfer of melanin containing melanosomes. Seiberg *et al.*, showed that activation of the protease activated receptor-

2 (PAR-2), which is expressed only on keratinocytes, increases melanin transfer to keratinocytes (Seiberg *et al.*, 2000).

1.1.4 Role of melanocytes:

- Melanocytes are responsible for skin, hair and eye color by manufacturing melanin in melanosomes and mediating their transfer to neighboring cells via dendritic processes.
- Melanocytes are responsible for sun tanning, as UV irradiation leads to increased melanin production and transfer of melanosomes to keratinocytes to protect against UV-induced DNA damage.
- Melanocyte stem cells reside in the bulge of the hair follicles and loss of the stem cell population gives hair graying.
- Melanocytes located in the stria vascularis are required for hearing and their loss can lead to deafness. (Hearing *et al.*, 2000).

1.1.5 Melanocyte immunobiology:

Melanosomes contain enzymes and structural components required for effective melanization. MART-1 appears to lay the groundwork for the deposition of the melanosomal matrix, assisted by gp100 (Hoashi *et al.*, 2005). These two molecules are also considered as the most immunogenic antigens of the melanocytic cells and T cells infiltrating melanoma tumors are most frequently reactive with either of these antigens (Kawakami *et al.*, 1997).

The subsequent arrival of tyrosinase and tyrosinase related enzymes TRP-1 and TRP-2 completes the arsenal of melanosomal enzymes. The melanosome is unique in particular because it is transferred to neighboring cells in a process that remains poorly understood till date. This process is of particular interest to immunologists. Organelle transfer has otherwise been described only for exosomes, small lipid vesicles serving to discard or transfer membrane proteins. Exosomes are considered important for Dendritic Cell activation and the induction of immune responsiveness to antigens contained within these organelles (Taieb *et al.*, 2005). Interestingly,

organelles transferred from melanocytes to keratinocytes are in fact equivalent to lysosomes in other cells. It is well possible that lysosome transfer can likewise occur between cells as well but cannot be easily observed for lack of a marker as readily detectable as melanin. The transfer of lysosome-like organelles may allow for sharing of antigens destined for presentation in the context of MHC class II. It should be noted that ripe melanosomes do not contain intact melanogenic enzymes as shown by melanocyte fractionation (Orlow *et al.,* 1993). But peptide fragments may be generated within the acidic environment of the melanosome. Resulting peptides may retain their immunogenicity when transferred to dendritic cells. Thus peptides derived from gp100, MART-1 and other melanosomal proteins, may be transferred to infiltrating dendritic cells in the absence of melanocyte death within the skin.

Melanocyte immunogenicity has also been ascribed to their Langerhans cell-like morphology and distribution. Melanocytes synthesize primary cytokines, and are thus capable of eliciting a local immune response (Kruger-Krasagakes *et al.,* 1995). Moreover, melanocytes are capable of phagocytosis and can process and present antigens in the context of MHC class II to $CD4^+$ proliferative and cytotoxic T cells (Le Poole *et al.,* 1993, Le Poole *et al.,* 1993). As melanocytes lack the mobility of Langerhans cells and other professional antigen presenting cells, it is conceivable that antigen presentation within the skin renders the melanocyte a 'sitting duck' for an immune attack. Of course, this situation will arise only when the melanocyte expresses MHC class II molecules, which happens to be the case only in vitiligo and in melanoma (Overwijk *et al.,* 2000).

1.2 TYPES OF VITILIGO

Vitiligo is most often classified clinically according to the extent and distribution of depigmentation (Figure 4) (Gawkrodger et al., 1998). It has been proposed that the segmental and focal presentations of the disease constitute a separate subgroup to the non-segmental forms of vitiligo (Taieb et al., 2000) because, compared with focal and segmental vitiligo non-segmental forms show a later age of onset, a stronger association with autoimmunity and unstable results following autologous grafting.

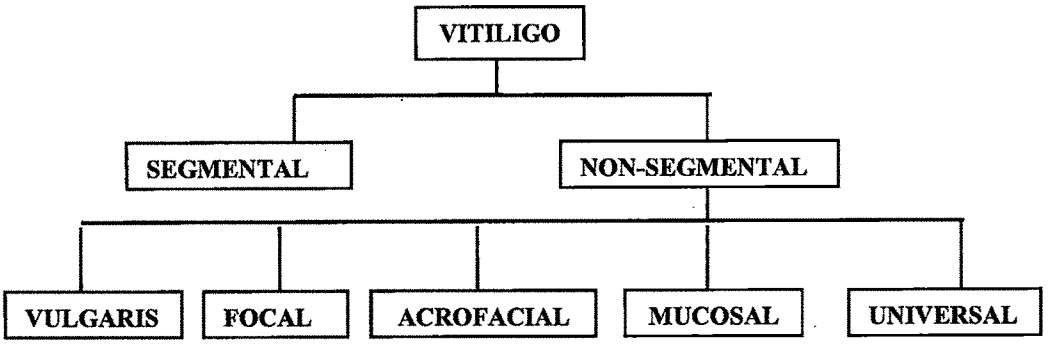

Figure 4: Classification of vitiligo (Dave *et al.*, 2002).

1.2.1 Segmental/unilateral-

It is often present in childhood and occurs in dermatomal, asymmetric distribution with one or more macules localized to one area of the body. It is rarely associated with autoimmune disorders and responds well to autologous grafting (Kemp *et al.*, 2001).

1.2.2 Focal vitiligo-

It describes one or more depigmented patches localized in a discrete area with autoimmune disorders and responds well to autologous grafting (Kemp *et al.*, 2001).

1.2.3 Symmetrical/bilateral vitiligo-

This is the most common type of vitiligo and is often referred to as generalized. It is characterized by a bilateral, symmetrical depigmentation with a widespread distribution of many macules in a random pattern. Many parts of body can be affected, including the face (particularly periorificial areas), the neck, torso, hand and legs. A latter age of onset is normal for this clinical subclass and it is often associated with autoimmunity. Unstable results are evident following autologous grafting in patients with this type of vitiligo (Kemp *et al.*, 2001).

1.2.4 Acrofacial vitiligo-

Vitiligo of this type is characterized by depigmentation of the distal fingers and facial orifices- the latter in a circumferential pattern. It often forms with symmetrical vitiligo (Kemp *et al.*, 2001).

1.2.5 Universal vitiligo-

This type of vitiligo is characterized by loss of pigmentation over the entire body, but is rare (Kemp *et al.*, 2001).

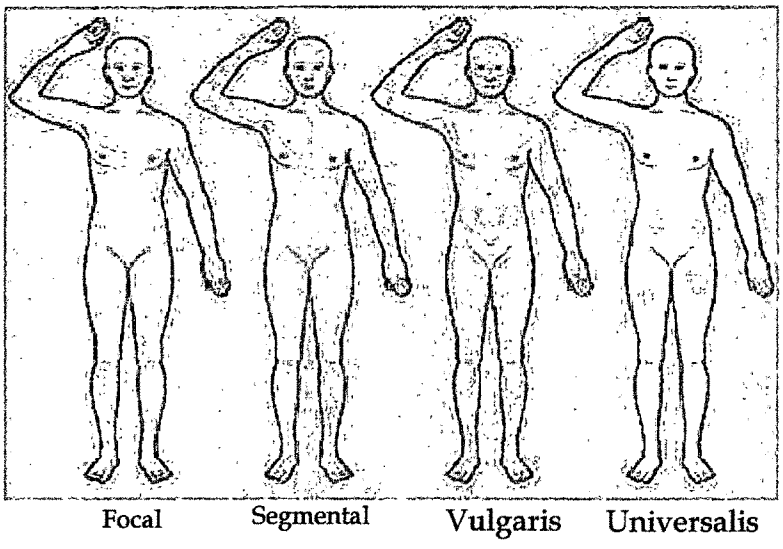

Figure 5: Clinical types of vitiligo

1.3 Causes of vitiligo:

Vitiligo involves complex interaction of environmental and genetic factors that ultimately contribute to melanocyte destruction, resulting in the characteristic depigmented lesions (Spritz, 2008).There is a gradual loss of melanin pigment from the skin layers which results in white patches. It is difficult to precisely define the triggering factors for vitiligo. Nevertheless, it is essential to elicit the details of the history of emotional stress, drug intake, infections, trauma/injury (Koebner phenomenon) existent prior to the development of vitiligo lesions. Figure 6 shows various probable triggering factors in the natural history/ development of vitiligo. A better understanding of these factors may prove to be helpful in the management of vitiligo.

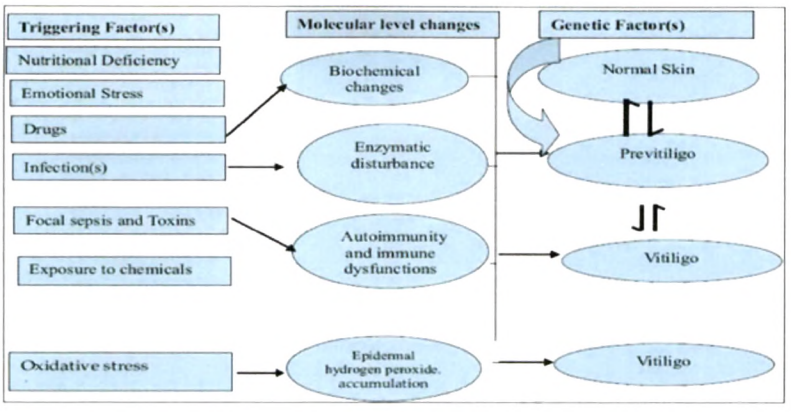

Figure 6: The triggering factors in vitiligo.

1.4 Pathogenesis of vitiligo:

Vitiligo is an idiopathic disease. Many triggering factors for vitiligo have been proposed. As patients are not born with the disease, it is thought that an initiating event, such as illness, stress, UV exposure or injury may trigger depigmentation.
Various hypotheses have been proposed explaining the etiology of vitiligo and the most important theories are:

- Oxidative stress
- Neurochemical
- Autoimmune
- Genetic

It is possible that these different causal factors can act independently or together to yield the same effect i.e. disappearance of melanocytes from the skin (Le Poole *et al.*, 1993).

1.4.1 Oxidative Stress and Vitiligo:

Oxidative stress is a major form of assault on the skin. Skin exposure to ionizing and UV radiation and/or xenobiotics/drugs generates ROS in excessive quantity. It overwhelms tissue antioxidants and other oxidant-degrading pathways. Uncontrolled release of ROS and increased lipid peroxidation are seen in multiple skin disorders such as atopic dermatitis, psoriasis, acne vulgaris, pemphigus vulgaris (PV), lichen

planus, alopecia areata and vitiligo (Yesilova *et al.*, 2012). Oxidative stress has been implicated in the initial pathogenic event in melanocyte destruction (Schallreuter *et al* 1999a; Maresca *et al.,* 1997) marked by accumulation of H_2O_2 in the epidermis of vitiligo patients (Schallreuter *et al.*, 1999a; Schallreuter *et al.*, 2001). Defective recycling of tetrahydrobiopterin in vitiligo epidermis is related with the intracellular production of H_2O_2 (Schallreuter *et al* 1994; Schallreuter *et al.*, 1999b). In addition, a compromised antioxidant status with a significant reduction in catalase activity and increase in SOD activity has been demonstrated in both lesional and non-lesional epidermis (Schallreuter *et al.*, 1991; Sravani *et al.*, 2009) as well as in melanocytes (Maresca *et al.*, 1997). Interestingly, antioxidant imbalance has also been observed in the peripheral blood mononuclear cells of active vitiligo patients. This was correlated with an increased intracellular production of reactive oxygen species due to mitochondrial impairment (Dell'Anna *et al.*, 2001), supporting the concept of a possible systemic oxidative stress in vitiligo.

In normal cellular processes, O_2^- radicals are converted into H_2O_2 due to the action of SOD enzyme which in turn converted to oxygen and water by the action of catalase and glutathione peroxidase. As a result cell maintains its normal integrity and function. Sometimes equilibrium of antioxidants and oxidants gets imbalanced which leads to accumulation of free radicals resulting into accumulation of oxidative stress (Figure 7). This accumulated oxidative stress causes DNA damage (Mohamed and Salem, 2009), lipid and protein peroxidation .Many proteins and peptides, results in altered or even complete loss of functionality due to H_2O_2-mediated oxidation.

The inhibition of thioredoxin reductase, a free radical scavenger located in the membrane of melanocytes, also seems to contribute to the generation of oxidative stress in the vitiligo epidermis (Schallreuter and Pittelkow, 1988). Further, increased amount of superoxide radicals generated due to elevated levels of extracellular calcium levels has been shown to inhibit tyrosinase (Schallreuter *et al.*, 1996b). Several sources have been documented for the unusual production/accumulation of epidermal H_2O_2 (Passi *et al.*, 1998; Schallreuter *et al.*, 1991; 1996a; 1999a; Rokos *et al.*, 2002). Since even millimolar levels of H_2O_2 can inactivate catalase (Aronoff 1965; Schallreuter *et al.*, 1991; Dell'Anna *et al.*, 2001) and glutathione peroxidase (Beazley

et al., 1999; Agrawal *et al.*, 2004), such high levels of accumulation is likely to have detrimental effects (Figure 8, pathways 1,2 and 4). Contextually, higher concentration of H_2O_2 by its inhibitory effect on 4 α-carbinolamine dehydratase is shown to disrupt recycling of the essential cofactor (6R)- L - erythro- 5,6,7,8 tetrahydrobiopterin ($6BH_4$) for aromatic amino acid hydroxylases as well as nitric oxide synthases (Hasse *et al.*, 2004). Further, Giovannelli *et al.*, 2004 have shown significantly higher level of oxidative DNA damage in mononuclear leukocytes in active vitiligo patients compared to controls.

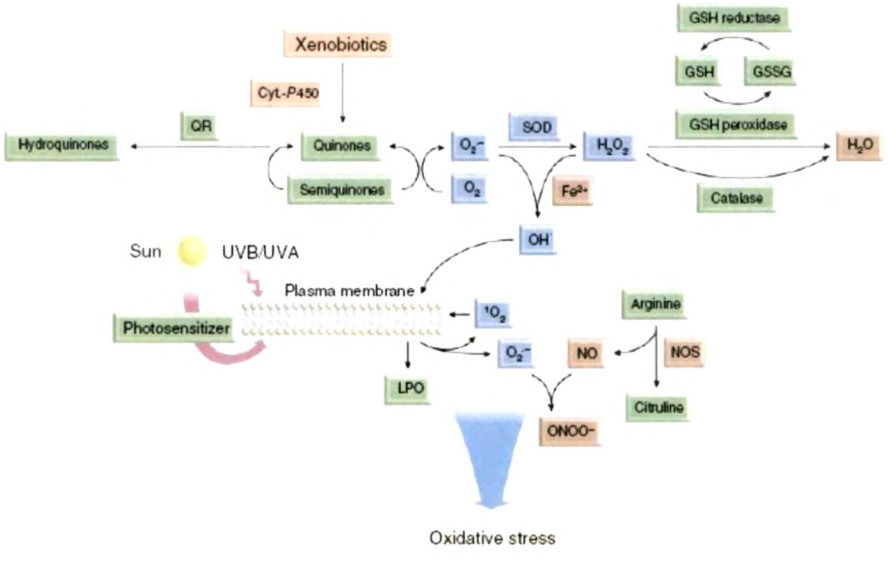

Figure 7: Reactive oxygen species generation leading to oxidative stress.

We have reported systemic oxidative stress in vitiligo patients due to an imbalance in enzymatic and non-enzymatic antioxidant systems (Agrawal *et al.*, 2004). Our observations suggest different mechanisms of generation of oxidative stress in different clinical types of vitiligo (Shajil and Begum, 2006). Whereas low levels of catalase may contribute to the generation of oxidative stress in segmental vitiligo, generation of oxidative stress in non-segmental vitiligo appears attributable to lower levels of glutathione peroxidase and reduced glutathione (Shajil and Begum, 2006). Moreover, our studies on neurochemical basis of vitiligo have documented significantly decreased acetylcholine esterase (AChE) activity (Shajil *et al.*, 2006). This could be due to H_2O_2 mediated oxidation of AChE, which emphasizes the role of oxidative stress in precipitating vitiligo (Schallreuter *et al.*, 2004). Natarajan *et al.*, 2010 have also shown the involvement of Nrf2-dependent Phase II detoxification

pathway, considering its importance in the regulation of epidermal skin homeostasis. The role of enhanced Nrf2 effectors seems to be the prime protective function conferred by this pathway on skin homeostasis. Apart from the protective response, phase II genes could also be interestingly responsible for sustaining depigmented lesions in vitiligo patches. Thus, this study demonstrated the involvement of Nrf2 and phase II genes in homeostatic mechanisms of vitiligo skin and moreover, lesional skin from vitiligo patients recorded higher levels of oxidative stress compared to non-lesional pigmented skin (Natarajan *et al.*, 2010). Eskandani *et al.*, 2010 have reported increased DNA damage in leucocytes and lowered levels of tyrosinase activity in lesional skin of vitiligo patients compared to their non-lesional skin. Tyrosinase is an important sensitive enzyme in pigmentation process as a range of factors can influence its activity including oxidative agents such as H_2O_2. In presence of DOPA substrate and 3-methylbenzothiazolinone-2-hydrazone (MBTH), H_2O_2 can function as an inhibitor of tyrosinase or, the presence of H_2O_2 and DOPA substrate can generate a secondary complex that can bind and inhibit the enzyme. This study suggested a meaningful correlation between increased oxidative stress and decreased tyrosinase activity (Eskandani *et al.*, 2010). In addition, an important role for oxidative stress in pathogenesis of vitiligo in experimental mice, suggesting that melanocyte damage in vitiligo might be linked to generalized oxidative stress (Jalel *et al.*, 2009).

Thus high ROS/RNS in melanocytes may cause the cell to undergo defective apoptosis and release of aberrated proteins from the cell. Failure of phagocytosis of these apoptotic cells causes expression of auto-antigens. These auto-antigens are presented by MHC to T-cells leading to autoimmunity (Kühtreiber *et al.*, 2003). TNF-α is known as a paracrine inhibitor of melanocyte growth and increased levels of TNF-α cause maturation of dendritic cells and thus results in to development of autoimmunity (Clemens *et al.*, 2000). The intracellular levels of H_2O_2 and other ROS also increase in several cellular systems in response to external stimuli and cytokines such as TNF-α and TGF β1 (transforming growth factor β1) (Celia *et al.*, 2001). High ROS also increases the levels of cytokines, including IL-2 which upregulates the expression of anti-apoptotic protein, Bcl-2 thereby making T-cells resistant to apoptosis (Figure 9, pathway 2). These cytokines are potent inhibitors of melanogenesis in B16 melanoma cells and human melanocytes (Swope *et al.*, 1991; Martínez-Esparza *et al.*, 1997; Martínez-Esparza *et al.*, 1998). In addition, the

generation of ROS/RNS and/or the resulting increase in lipid peroxidation products have been proposed for hair graying (Nordlund and Abdel-Malek, 1988) and several pathological conditions, like vitiligo (Passi *et al.*, 1998).

1.4.2 Neural Factors and vitiligo:

Melanocytes are neural crest derived cells giving them an embryological link to the nervous system (Reedy *et al.*, 1998). Neurochemical mediators that are secreted by the nerve endings such as norepinephrine and acetylcholine are toxic to melanocytes (Figure 8, pathway 3). Different studies on vitiligo patients showed higher levels of plasma and urinary catecholamines and their metabolites especially at the onset and in the active stage of the disease (Morrone *et al.*, 1992; Orecchia *et al.*, 1994; Cucchi *et al.*, 2000; Cucchi *et al.*, 2003). An increased release of catecholamines from the autonomic nerve endings in the microenvironment of melanocytes causes melanocyte death and ultimately resulting in to vitiliginous lesions (Figure 8, pathway 3).
Norepinephrine has both direct and indirect melanocytotoxic actions which include its interaction with cellular sulfhydryl groups, enzyme inhibition, impairing mitochondrial calcium uptake, and forming some cytotoxic products including free radicals (Figure 9, pathway 1). High concentrations of norepinephrine and its metabolites in vitiligo patients may be due to a reduction in phenylethanolamine-N-methyl transferase (PNMT) activity and an increase in tyrosine hydroxylase (TH) activity (Schallreuter *et al.*, 1994). These enzymes play a key role in production of L-DOPA form, L-tyrosine. Also (6R)-5,6,7,8-tetrahydrobiopterin (6BH4), the rate limiting cofactor/electron donor for TH is increased due to decreased 4a-hydroxy-6BH4 dehydratase (DH) activity in vitiligo patients (Schallreuter *et al.*, 2001). There is also a defective recycling of $6BH_4$ which leads to increased non-enzymatic production of $7BH_4$, an isomer, concomitant with an increased production of H_2O_2. Presence of $7BH_4$ in the epidermis seems to initiate the process of depigmentation in vitiligo patients by blocking the supply of L-tyrosine to melanocytes. These alterations seem to cause melanocyte destruction in vitiligo (Schallreuter *et al.*, 1994). The indirect effects include activating α-receptors of the arterioles, causing a severe vasoconstriction, and thereby producing toxic oxygen radicals caused by hypoxia. Increased levels of norepinephrine also appear to induce another catecholamine degrading enzyme, monoamine oxidase (MAO) (Bindoli *et al.*, 1992). Keratinocytes

and melanocytes in the depigmented skin exhibit increased monoamine oxidase-A activity which further causes keratinocytes to produce 4-fold more norepinephrine and 6.5-fold less epinephrine than control keratinocytes (Schallreuter *et al.*, 1996a). Norepinephrine is reported to be toxic to melanocytes. The increased MAO-A activity favors the formation of hydrogen peroxide, which is toxic to melanocytes (Schallreuter *et al.*, 1996a). Moreover, the induced damage to melanocytes is not buffered by low epidermal catalase levels (Schallreuter *et al.*, 1991).

A derangement of the enzyme dealing with catabolism of adrenergic transmitters namely catechol-o-methyl transferase (COMT) is also reported. COMT normally prevents the formation of toxic ortho quinones during melanin synthesis. Vitiligo patients show higher epidermal COMT activity, probably induced in the tissues by elevated levels of catecholamines secreted by keratinocytes or by nerve endings (Le Poole *et al.*, 1994).

In addition, neurochemical mediators such as acetylcholine secreted by the nerve endings cause the destruction of melanocytes. Acetylcholine esterase activity is found to be lowered in vitiliginous skin during depigmentation (Iyengar, 1989). Our study also reported decreased acetylcholine esterase activity in vitiligo patients and suggested that acetylcholine esterase may be inactivated due to high systemic oxidative stress in these patients (Shajil *et al.*, 2006).

Aberrations in beta-endorphin and met-enkephalin secretion are also reported in vitiligo patients (Mozzanica *et al.*, 1992). In vitiligo, the levels of met-enkephalin levels are found to be higher. It is suggested that this abnormality may be correlated with the emotional stress, which precipitates vitiligo in some patients. Abnormalities of neuropeptides are observed in perilesional skin and blood of vitiligo patients (Al'Abadie *et al.*, 1994). The neuropeptide Y (NPY) is released by either exogenous stimulus like trauma (e.g. Koebner phenomenon) or by endogenous stimuli (e.g. stress) (Al' Abadie *et al.*, 1994) and this altered balance of neuropeptides in vitiliginous skin supports a role for the nervous system in the pathogenesis of vitiligo (Liu *et al.*, 1996). Neuropeptides are also reported to have immunoregulatory effects (Covelli and Jirillo, 1988). Caixia *et al.*, 1999 showed that the levels of NPY in the plasma of vitiligo patients were found to be significantly higher than the normal

controls. The levels of NPY from skin lesions were significantly higher than those from uninvolved skin in both the local type and segmental types of vitiligo. NPY could evoke the secretion of IFN- γ and IL- 2 (Figure 8, pathway 2) suggesting that NPY might be involved in the cell mediated immunological mechanism, which plays a role in the melanocyte destruction in vitiligo (Caixia *et al.*, 1999).

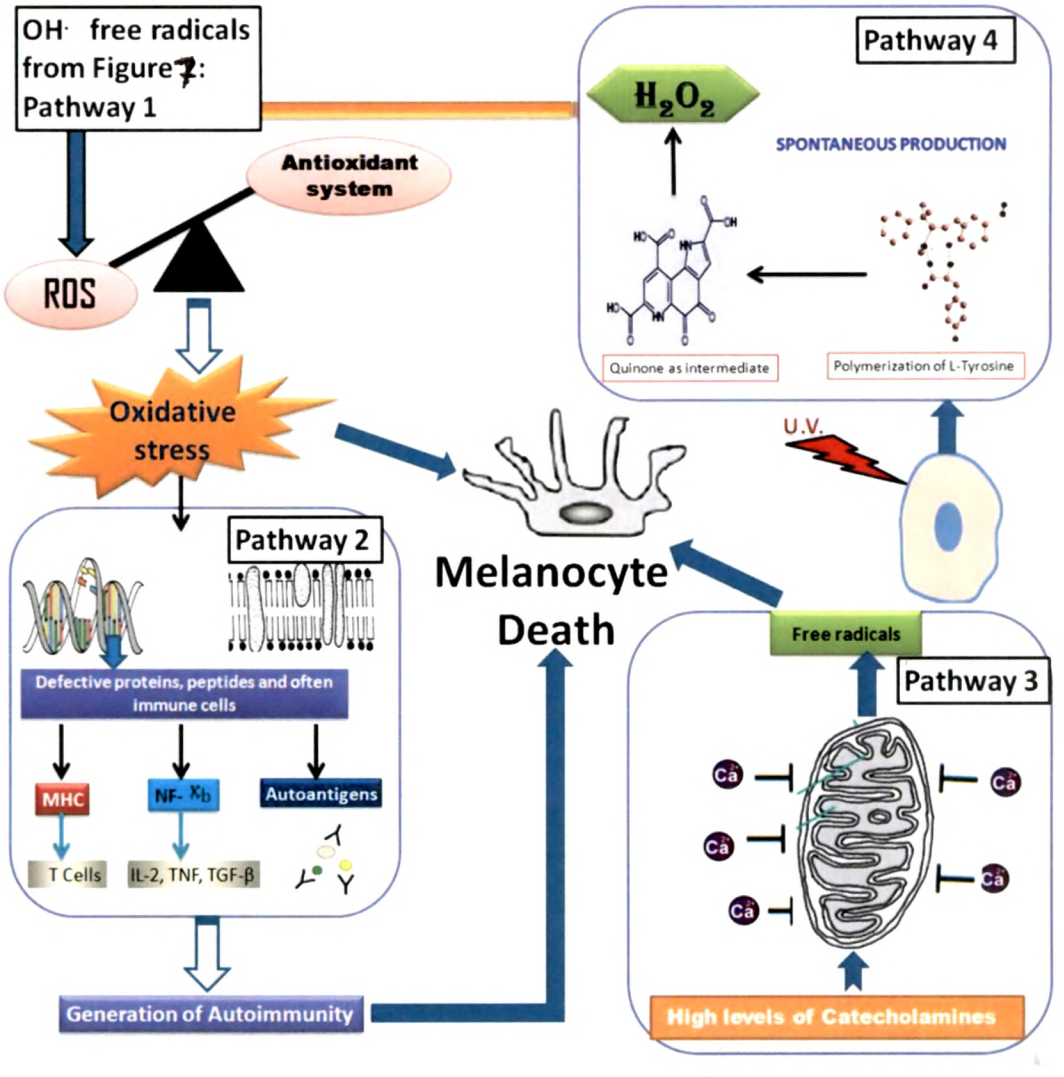

Figure 8: Different pathways for melanocyte destruction:

1. Generation of ROS by various metabolic processes. 2. Imbalance in ROS generation and antioxidant system leads to accumulation of free radicals resulting in oxidative stress. This accumulation causes DNA damage, synthesis of defective proteins and membrane disintegration which provokes immune system resulting in autoimmunity. 3. Increased catecholamines inhibits mitochondrial calcium uptake

which results in free radicals generation 4. Exposure to UV radiation, leads in spontaneous production of quinones in melanocytes which in turn results in ROS generation.

1.4.3 Autoimmunity and vitiligo:

Different hypotheses have been proposed to explain this disorder (Halder and Chappell, 2009). Nevertheless, there is strong evidence that vitiligo is preferentially an autoimmune disease. The association with autoimmune conditions such as Addison's disease, hypothyroidism, pernicious anemia and systemic lupus erythematosus; presence of some alleles of MHC II antigens and other autoimmune-susceptibility genes; detection of organ-specific antibodies (anti-ANA, anti-gastric parietal cells) in the serum of patients with vitiligo; positive response to topical immunosuppressant therapy (topical steroids and tacrolimus); studies in animal models of vitiligo; the participation of immune cells in the demonstration of auto reactive T cells; and the presence of antibodies against different antigens of melanocytes; all these aspects support autoimmune hypothesis (Ongenae *et al.*, 2003; Passeron and Ortonne, 2005). It has also been proposed that two major mechanisms are related to autoimmune vitiligo, an antibody-based dominant mechanism in diffuse vitiligo and a T-cell-based dominant mechanism in localized disease (Michelsen, 2010). Therefore, the role played by cellular and humoral immune responses in the pathogenesis of vitiligo has been further discussed.

1.4.3.1 Autoantibodies and antigens:

Different circulating antibodies to melanocytes, which are uncommon in healthy individuals, have been found in the sera of vitiligo patients. These antibodies seem to be related with the extent of disease, and are present in more than 90% of the patients with greater depigmentation and in 50% of the patients with minimal lesions (Abu Tahir *et al.*, 2010). Various methods have been used for their detection. Characterization of these antibodies has demonstrated that they belong to the IgG class, involving the subclasses IgG1, IgG2 and IgG3. Interestingly, IgG and C3 deposits have also sporadically been observed in the basal membrane zone of lesional skin, which correlates with the observation that the binding of IgG to cultured

melanocytes increases with disease activity and extent. Furthermore, studies have found that IgA levels of anti- pigment cell membrane antibodies are associated with disease activity, suggesting a close relationship with anti-melanocyte IgA antibody levels (Ongenae *et al.*, 2003; Kemp *et al.*, 2007). These findings together, may be linked to the alterations in immunoglobulins showed in vitiligo. A recent study of the serum immunoglobulin profile of vitiligo patients showed that IgG and IgA levels are significantly decreased ($p=0.05$) with no changes in IgM, compared to controls (Ali *et al.*, 2010), indicating that IgG anti-melanocyte antibodies could have an important role in this disorder, as they can induce melanocyte damage *in vitro* by a complement-mediated mechanism and antibody-dependent cellular cytotoxicity. In addition, IgG anti-melanocyte antibodies may play an active role in the stimulation and inappropriate expression of HLA-DR and induction of ICAM-1 on melanocytes, and also an increased IL-8 production. Thus, MHC II molecules expressed on melanocytes can present antigens to $CD4^+$ cells, allowing an immune response, and ICAM-1 may play an essential part in immunological and inflammatory reactions resulting in melanocytotoxicity (Li *et al.*, 2000). Ruiz- Argüelles *et al.* (2007), studied 15 patients with vitiligo and found that 93% patients showed antibodies to a 75 kDa melanocyte protein in serum samples, and apoptotic markers in vitiligo skin biopsies. When melanocytes were cultured with purified IgG from vitiligo patients, apoptosis was induced and the cells showed a higher proportion of intracellular IgG suggesting that antibodies directed to melanocyte-specific antigens may enter the melanocytes and induce apoptosis.
A few specific autoantigens identified include tyrosinase, a melanocytic enzyme; tyrosinase-related protein (TRP) 1 /gp75 and 2; the melanosomal matrix protein gp100 (Pmel17) and Melan A/MART 1 etc. as shown in Table 1.

In addition, a recent study using phage display identified B-cell autoantigens i.e., antigens to gamma-enolase (8%), alpha-enolase (9%), heat-shock protein 90 (13%), osteopontin (4%), ubiquitin-conjugating enzyme (15%), translation-initiation factor 2 (6%), and GTP-binding protein, Rab38 (15%), in patients with non-segmental vitiligo while in contrast, patients with segmental vitiligo had no reactivity to these autoantibodies (Waterman *et al.*, 2010). However, none of these proteins have been identified as the major autoantigen in vitiligo.

Table 1: Antigens recognized by autoantibodies of vitiligo patients.

Autoantigens	Reference
Tyrosinase	Song *et al.*, 1994, Baharav *et al.*, 1996; Xie *et al.*, 1999; Kemp *et al.*, 1997
TRP 1	Kemp *et al.*, 1998b
TRP 2	Okamoto *et al.*, 1998; Kemp *et al.*, 1997
Pmel 17	Kemp *et al.*, 1998a
Melan A/MART 1	Waterman *et al.*, 2002
MCHR 1	Waterman *et al.*, 2002
SOX 9	Hedstrand *et al.*, 2001
SOX 10	Hedstrand *et al.*, 2001

1.4.3.2 T cell participation:

Skin biopsies of vitiligo patients have shown that inflammatory cells are prominent in perilesional areas, and one of the first clues for the participation of cellular immunity in the pathogenesis of vitiligo was the discovery of T cell infiltration in the margins of active vitiligo skin lesions associated with areas of melanocyte loss. This perilesional infiltration consists of $CD8^+$ and $CD4^+$ T cells, often with an increased $CD8^+/CD4^+$ ratio (Le Poole *et al.*, 2004). An *in vitro* study analyzing T cells from perilesional and nonlesional skin biopsies from vitiligo patients found that both $CD8^+$ and $CD4^+$ T cells in perilesional areas exhibit a predominantly Type-1-like cytokine secretion profile, with secretion of TNF-α and IFN-γ. In particular, IFN-γ enhances T-cell trafficking to the skin by increasing ICAM-1 expression (Wańkowicz-Kalińska *et al.*, 2003). Another important observation is expression of the cutaneous lymphocyte-association antigen (CLA) by $CD8^+$ cells, which is a skin homing receptor that could recruit T cells from peripheral circulation to the affected skin (Ogg *et al.*, 1998). High frequencies

of Melan A/MART 1 specific $CD8^+$ T cells in perilesional skin and peripheral blood have been detected; these cytotoxic T cells showed *in vitro* anti-melanocyte cytotoxic activity and expressed skin-homing capacity, which seems to correlate with disease extension and severity (Palermo *et al.*, 2001). Van den Boorn *et al.* (2009) in a study with a skin explant model, investigated *in situ* activities of perilesional $CD8^+$ T cells in the effector phase of depigmenation and found that $CD8^+$ T cells could infiltrate normally pigmented skin explants and eradicate melanocytes. In the same study, melanocyte apoptosis was accompanied by suprabasal keratinocyte apoptosis, and perilesional cytotoxic T cells did not induce apoptosis in lesional skin devoid of melanocytes, which indicates their melanocyte-specific cytotoxic activity and skin- homing capacity. The capacity of cytotoxic cells for melanocyte damage has also been observed in an experimental murine model of vitiligo. It was found that melanocytes can be destroyed by $CD8^+$ T cells that recognize a single H2-Kb-binding peptide derived from tyrosinase-related protein 2 (TRP-2) (Steitz *et al.*, 2004). Additionally, the presence of granzyme B and perforin expressed by CLA/HLA-DR/$CD8^+$ T cells at the perilesional epidermal–dermal junction of vitiliginous skin shows the major role of T cells in melanocyte death in vitiligo (van den Wijngaard *et al.*, 2000; Oyarbide-Valencia *et al.*, 2006).Moreover, it is proposed that the patchy, symmetrical distribution of lesions is caused by autoimmune melanocyte damage due to clones of lymphocytes with affinities for specific skin areas (al Badri *et al.*, 1993). This reactivity of $CD8^+$ T cells towards melanocytes observed in vitiligo patients could be used in the clinical application in the treatment of melanoma (Oyarbide-Valencia *et al.*, 2006).

Autoimmune diseases are often associated with malfunction of peripheral $CD4^+$ cells, and therefore $CD4^+$ T cells may be involved in the pathogenesis of vitiligo. It is suggested that peripheral tolerance of $CD8^+$ cells recognizing melanocyte self- antigens are regulated in two steps: firstly, an induction phase where the stimulation and expansion of autoreactive $CD8^+$ cells depend on $CD4^+$ T cells in the lymphoid system and, secondly, an effector phase where autoimmune destruction of pigmented cells depends on local inflammation, with the migration of T cells supporting the effector functions (Steitz *et al.*, 2005). Severe autoimmune vitiligo may develop after genetic removal of the cytotoxic T lymphocyte-associated

antigen 4 (CTLA-4), an important immunomodulatory molecule to the T cell tolerance expressed in $CD8^+$ and $CD4^+$ T cells. It has been shown that removal of CTLA-4 induces the activation and acquisition of effector functions in $CD8^+$ cells, but only in the presence of dysregulated $CD4^+$ T cells (Gattinoni *et al.*, 2006). Furthermore, a correlation between idiopathic $CD4^+$ T lymphocytopenia (ICTL) and autoimmune vitiligo has been reported, indicating that an abnormal response of $CD4^+$ T lymphocytes is involved in this disease (Yamauchi *et al.*, 2002). Abnormal expression of MHC class II molecules by perilesional melanocytes and increased numbers of expressed intercellular adhesion molecule ICAM-1, which play an important part in antigen presentation and $CD4^+$ T cell activation, have been documented (al Badri *et al.*, 1993). Likewise, studies have indicated that marginal skin from patients with progressive generalized vitiligo shows a down-regulated expression of CDw60 molecule by $CD4^+$ T cell type 2 (Le Poole *et al.*, 2003) and recent study of the polymorphism of the $CD4^+$A4 allele has shown a modest association with the development of vitiligo (Zamani *et al.*, 2010).

The hypothesis that depigmentation can progress in the absence of regulatory T cells (Treg) is established. Reduced numbers of Treg cells appear in non-lesional, perilesional and lesional vitiligo skins, and a reduced expression of the skin homing chemokine CCL22 in vitiligo skin has been observed by immunohistochemistry. This may explain the failure of circulating Treg cells and their reduction of skin homing due to the failure of functionality, which might perpetuate reactivity against melanocytes in vitiligo (Klarquist *et al.*, 2010).

Abnormalities in circulating T cells have been reported with inconsistent results, and there is no simple explanation for the differences. T cells have been reported to be normal, decreased or increased, and which may be attributable to various factors including the study techniques used to determine T-cell subpopulations, disease characteristics, the study population and previous therapy. A recent study by Pichler *et al.* (2009) evaluating lymphocyte fractions from peripheral blood in a cohort of patients with vitiligo found an elevated $CD4^+/CD8^+$ T cell ratio with a disturbed balance of cytotoxic/suppressor and helper/inducer T cells in peripheral blood. However, the absolute and relative counts of lymphocyte subtypes were normal. Another study found significantly

increased $CD3^+$ counts in generalized vitiligo and increased memory ($CD45RO^+$) T cells in acrofacial vitiligo compared with other vitiligo types, and raised percentages of $CD8^+$ T cells in vitiligo patients compared with controls (Basak *et al.*, 2008). Other studies have found decreased percentages of $CD4^+$ T cells (Grimes *et al.*, 1986), lower total lymphocytes, increased percentages of activated ($CD3^+$ HLA-DR^+) T cells and decreased total naive T cells ($CD4^+CD45RA^+$) (Mahmoud *et al.*, 2002) in vitiligo patients. Taken together, these findings suggest that an important disturbance of cell-mediated immunity takes place in this disorder. These are also situations where T cell responses to melanocytes prevail. In conclusion, a role for melanocytes within the Skin Immune System cannot be overlooked as we assess the etiology of vitiligo. However, the reasons for these inconsistent findings are not clear and require further study.

Skin infiltrating T cells can be isolated from tissue biopsies of interest and grown in bulk. T cells isolated from vitiligo skin are highly reactive with melanoma-associated antigens (Das *et al.*, 2001). High affinity TCRs, once expressed in PBMC from melanoma patients, can more effectively target the tumor (Duval *et al.*, 2006). It is well possible that high affinity T cells previously escaped clonal deletion in skin-draining lymph nodes and were inadvertently allowed to enter the circulation, emigrating to the skin to inflict damage to the melanocyte population (Palermo *et al.*, 2005). An ongoing autoimmune response may be allowed to further develop and mature in the absence of functional Tregs (Dejaco *et al.*, 2006). These cells actively mediate suppression of the immune system generally by secreting IL-10 and TGF-β to prevent autoimmunity (Dejaco *et al.*, 2006). In the absence of regulatory T cells, cytotoxic T cells with increasing affinity for their targets enter the skin and continuously proliferate and migrate towards novel target cells, causing depigmentation. This unfortunate event of disfiguring depigmented skin patches left in vitiligo patients may turn out to be a blessing in disguise if the T cell receptors expressed by high affinity T cells provide an effective treatment for melanoma patients.

1.4.3.2.1 Regulatory T cells (Tregs) in Autoimmune Diseases:

No definite surface marker is currently available for human Tregs. The high constitutive surface expression of the IL-2 receptor alpha chain (CD25) is generally

considered as a characteristic feature of the majority of human Tregs, and regulatory activity is enriched in $CD4^+$ T cells expressing the highest levels of CD25 ($CD4^+$ $CD25^{hi}$ T cells) (Baecher-Allan *et al.,* 2001; de Kleer *et al.,* 2004; Makita *et al.,* 2004; Cao *et al.,* 2003; Baecher-Allan *et al.,* 2005). Upon activation of T cells and independently of their regulatory capacity, most of these markers become up-regulated and have therefore only limited specificity to identify Tregs (Shevach *et al.,* 2004).

Intracellular expression of FOXP3 is currently considered as the most specific marker for human Tregs (von Boehmer *et al.,* 2005; Picca *et al.,* 2005; Sakaguchi *et al.,* 2005). Human FOXP3 is localized on the X chromosome encoding 'scurfin', which binds to the IL-2 promotor and the granulocyte–macrophage colony-stimulating factor enhancer near the nuclear factor of activated T cell (NFAT) sites. FOXP3 represses these genes, thus reducing IL-2 production by $CD4^+$ T cells. From the clinical perspective, a mutation of this transcription factor is strongly linked to immune dysregulation. Patients with a mutated FOXP3 gene encounter autoimmune polyendocrinopathy (especially type 1 diabetes mellitus and hypothyroidism) and enteropathy [immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome] (Wildin *et al.,* 2002). Studies have all supported the role of FOXP3 as a 'master control gene' in the development and functioning of Tregs (Sakaguchi *et al.,* 2005).

1.4.3.2.1.1 Levels of Tregs in autoimmune diseases:

Although several efforts have been made to combine different surface markers for a more specific characterization of Tregs (Ruprecht *et al.,* 2005, Koenen *et al.,* 2005) gating on $CD4^+$ $CD25^{hi}$ T cells is usually preferred to define Tregs. The level of circulating $CD4^+$ $CD25^{hi}$ T cells out of the $CD4^+$ T cell pool in healthy humans ranges from 0.6% to 7.9%. In patients with autoimmune diseases, reduced levels of circulating $CD4^+$ $CD25^{hi}$ T cells were described, specifically in individuals with juvenile idiopathic arthritis (Cao *et al* ., 2004; de Kleer *et al.,* 2004), psoriatic arthritis (de Kleer *et al.,* 2004), hepatitis C virus (HCV) associated mixed cryoglobulinaemia (Boyer *et al.*,2004), autoimmune liver disease (Longhi *et al.,* 2004), systemic lupus erythematosus (Liu *et al.,* 2004, Crispin *et al.,* 2003) and Kawasaki disease (Furuno *et al.,* 2004). Lower levels of circulating $CD4^+$ $CD25^{hi}$ T

cells also correlate with a higher disease activity or poorer prognosis (de Kleer *et al.*, 2004, Boyer *et al.*, 2004, Longhi *et al.*, 2004, Crispin *et al.*, 2003, Furuno *et al.*, 2004). It has been proposed that the reduced levels may be caused by the impaired proliferation of peripheral $CD4^+$ $CD25^{hi}$ T cells, as observed *in vitro* (Viglietta *et al.*, 2004, Longhi *et al.*, 2004). Thereby, the balance between pro-inflammatory and regulatory T cells would be disturbed, leading to the breakdown of self-tolerance. However, further studies are required to confirm these results *in vivo* and to exclude any bias of these experiments owing to contamination with anergic effector T cells expressing high levels of CD25 (Shevach *et al.*, 2004).

1.4.3.2.1.2 Different developmental stages of Tregs and Tregs homing:

The observation that murine Tregs expressing FOXP3 constitute several phenotypically and functionally distinct subsets (Belkaid *et al.*, 2005, Fontenot *et al.*, 2005, Huehn *et al.*, 2004) led to the concept of developmental stages of Tregs. In brief, one subgroup of $CD4^+$ $CD25^+$ Tregs expresses high surface levels of CD62L and the chemokine receptor CCR7, and preferentially homes to antigen-draining lymph nodes (similarly to naive T cells), where they efficiently inhibit induction of inflammatory reactions (Fontenot *et al.*, 2005, Huehn *et al.*, 2004, Siegmund *et al.*, 2005).

1.4.3.2.1.3 Suppressive mechanisms of Tregs:

The mechanisms used by Tregs to suppress immune responses are still unresolved. In brief, inhibition is contact dependent, and transwell assays and supernatants of Tregs revealed no suppressive effects. After activation, human Tregs may directly kill activated $CD4^+$ and $CD8^+$ T cells in a perforin or granzyme dependent manner *in vitro* (Grossman *et al.*, 2004). Although evidence for such Tregs mediated cytotoxicity is lacking *in vivo*, the observation of patients with mutations in the perforin gene and who suffer from haemophagocytic lymphohistiocytosis (HLH) indicates a critical involvement of perforin in the regulation of immune responses (Stepp *et al.*,1999). These patients have an overactive immune system and immune responses to infections are not down-regulated after the cessation of an infection. Untreated HLH patients then develop end organ damage from lymphocyte infiltration and macrophage activation. Perforin-deficient mice develop a disease similar to HLH after exposure to viruses (Badovinac *et al.*, 2003). Perforin

polymorphisms may thus predispose to a prolonged activation of the immune system during infections, which could provoke the breakdown of tolerance. Another effector mechanism of suppression has been proposed with reverse signalling through cross-linking B7 (CD80 and CD86) on the cell surface of antigen presentin cells or activated T cells. This signalling is mediated by CTLA-4 expressed on Tregs (Birebent *et al.*, 2004). However, as murine Tregs with target deletion of genes encoding CTLA-4 are suppressive *in vitro*, a non-redundant role of CTLA-4 in the suppressive process is unlikely (Tang *et al.*, 2004). Whether cytokines modulate the T_{reg} mediated suppression of immune responses is also unclear. Both TGF-β and IL-10 have been linked to this effect in murine colitis and type 1 diabetes, although *in vitro* blockade of TGF-β or IL-10 does not totally abrogate suppression (Shevach *et al.*, 2002; Green *et al.*, 2003; Asseman *et al.*,1999).

The signals that facilitate or direct peripheral T_{reg} formation remain elusive, but may involve costimulatory molecules such as CTLA-4, cytokines such as TGF-β, and dendritic cells (Sakaguchi *et al.*, 2004; Sakaguchi *et al.*, 2005).

1.4.3.2.2 Imbalanced immune homeostasis and autoimmunity:

Normal maintenance of the homeostatic equilibrium is achieved through thymic T-cell generation with subsequent development of peripheral T cells and cell death. Under conditions of an extremely disturbed immune system, such as lymphopenia or acute depletion of lymphocytes, T cells undergo peripheral proliferation in the absence of foreign antigen stimulation and can restore the size of the peripheral T-cell compartment independently of the thymic output of naive T cells (Jameson *et al.*, 2002). As lymphopenic states are common throughout life, for example, during viral infections, lymphopenia-induced proliferation may be the primary mechanism to restore the T-cell pool in aged individuals with reduced thymic function (Khoruts *et al.*, 2005).

Although mechanisms that regulate this proliferation are still under investigation, it became apparent that the proliferative capacity of individual T cells correlates with their avidity for self-ligands (Khoruts *et al.*, 2001). Indeed, in patients with rheumatoid arthritis, a contraction of TCR diversity and oligoclonal T-cell expansion

further support this concept (Koetz *et al.*, 2000; Utsinger *et al.*, 1976; Rivero *et al.*, 1978; Iannone *et al.*, 1996; Heimann *et al.*, 1986; Wagner *et al.*, 1998).

1.4.3.2.3 Breakdown of tolerance by environmental factors:

Depletion of $CD25^+$ cells in mice eliminates a high proportion of circulating T_{regs} and activated T cells with severe lymphopenia, but is not sufficient to induce autoimmunity without administration of strong adjuvants. This observation supports the multifactorial pathogenesis of autoimmunity (McHugh *et al.*, 2002). Environmental factors, such as microbial infections or drug metabolism, have long been suspected to trigger the onset of autoimmune diseases by antigen-specific and non-specific effects. Molecular mimicry is one possible mechanism used by microbes to break immune tolerance (von Herrath *et al.*, 2003).The underlying concept is that infectious agents share one or more epitopes with various self components. An alternative hypothesis is that infectious agents cause bystander activation of immune cells with autoaggressive potential. Thus, infections cause transient lymphopenia and organ damage with a release of autoantigens, favouring the proliferation of T cells bearing an autoreactive TCR (Christen *et al.*, 2004). In addition, they provoke the presentation of self-antigens, together with cytokines and costimulatory molecules, as a 'danger' signal (Matzinger *et al.*, 1994).These danger signals are important for inducing an effective immune response against microbes by activating not only naive T cells but also attenuating Tregs mediated suppression (Takahashi *et al.*, 1998, Christen *et al.*, 2004, Horwitz *et al.*, 1998). In the event of disturbed tolerance mechanisms with impaired Tregs generation and bias of non-regulatory T cells towards autoreactivity, infections may initiate or even trigger 'pre-existing' autoimmunity. Genetic polymorphisms of molecules influencing Tregs generation or activation, such as IL-2, CTLA-4 or CD28 (Encinas *et al.*, 1999, Atabani *et al.*, 2005), the timing of infection and the magnitude of inflammation may be additional factors involved in the exacerbation of autoimmunity (Horwitz *et al.*, 1998).

1.4.3.2.4 Tregs -mediated therapeutic approaches for the future:

Strategies to support Tregs in autoimmune diseases are considered an intriguing new approach for using to suppress the inflammatory process, by manipulating both the function and number of Tregs. It is believed that protocols for such manipulation have

the therapeutic potential to induce tolerance in patients with autoimmune diseases, because in mice with collagen-induced arthritis, depletion of Tregs caused rapid progression, but early joint damage could be reversed by the transfer of isolated and *ex vivo* proliferated Tregs (Morgan *et al.*, 2003). Other animal models of autoimmunity show similar results (Kohm *et al.*, 2002). Monoclonally expanded Tregs, which specifically target autoantigens, may even provide more efficient suppression, (Tarbell *et al.*, 2004) and protocols for the *in vitro* expansion of human Tregs are already available (Hoffmann *et al.*, 2004). Alternatively, transfection of nonregulatory T cells with FOXP3 could generate antigen specific Tregs with increased suppressive activity that target sites of inflammation (Yagi *et al.*, 2004, Oswald-Richter *et al.*, 2004, Hori *et al.*, 2003). Transfection of polyclonally expanded Tregs with genes encoding TCR-α and β chains or genes encoding a chimeric antigen MHC-CD3-f molecule might also result in significant numbers of potent, highly directed, antigen specific Tregs (Mekala *et al.*, 2005).

Other approaches to increase Tregs function or numbers without *ex vivo* manipulation include administration of cytokines (such as TGF-β) that favour Tregs activity and survival. TGF-β may also convert non-regulatory T cells into Tregs (Wan *et al.*, 2005) and TGF-β gene therapy was found to induce Tregs in syngenic islet transplanted non-obese diabetic mice, blocking islet destructive autoimmunity (Luo *et al.*, 2005). Tolerance can also be induced by short-term treatment with monoclonal antibodies against costimulatory molecules, adhesion molecules or the TCR complex (Waldmann *et al.*, 1998, Lehmann *et al.*, 1997, Beyersdorf *et al.*, 2005). There are, however, technical difficulties regarding reliable identification of Tregs and the generation of sufficient numbers of Tregs for therapeutic purposes (Bluestone *et al.*, 2005). T cells with retroviral expression of FOXP3 have recently been shown to be less suppressive than freshly isolated $CD4^+$ $CD25^+$ Tregs (Allan *et al.*, 2005) and may therefore be insufficient to down-regulate autoimmunity in humans.

1.4.3.3 B cell participation:

The role of B cells in the pathogenesis of vitiligo is unclear, while some studies have found no B cell infiltration in vitiligo skin lesions (Le Poole *et al.*, 1996). A study found B cell infiltration in juxtaposition to depigmented zones, supporting

the idea that the autoimmune phenomenon is mediated by a humoral mediator or is local to some areas of skin, suggesting that the depletion of melanocytes is mediated by locally secreted products (Argüelles *et al.*, 2007). Interestingly, another study found that the MCHR1 is a B cell autoantigen in vitiligo with the production of antibodies to this receptor (Gavalas *et al.*, 2009), confirming its participation. Therefore, blocking of this receptor may alter the signalling pathway that regulates melanocyte function, affecting pigment cell behavior (Kemp *et al.*, 2007).

1.4.3.4 Cytokines:

Various studies have investigated the presence and role of cytokines in the depigmentation process of vitiligo. Increased serum levels of soluble IL-2R have been associated with vitiligo activity, indicating T cell activation. Specifically, soluble IL-2R are significantly higher in focal type vitiligo compared with segmental or generalized types, and are higher in patients with less than a year of disease duration (Yeo *et al.*, 1999). In addition, elevated production of IL-6, a cytokine that induces ICAM-1 expression, facilitating leukocyte–melanocyte interactions, and IL-8, an attracting cytokine to neutrophils by mononuclear cells in vitiligo patients has been found (Yu *et al.*, 1997). Another study found decreased serum levels of transforming growth factor-β), which can diminish maturation of T_{reg} cells. Likewise, a correlation with the extension of vitiligo and levels of IL-17 has been demonstrated (Basak *et al.*, 2009). Studies found a significantly higher expression of pro-inflammatory cytokines with an inhibitory effect on pigmentation, such as IL-6 and TNF-α, in lesional and perilesional skins in vitiligo patients, but a lower expression of melanogenic mediators such as granulocyte-monocyte colony stimulating factor (GM-CSF), basic fibroblastic growth factor (bFGF), stem cell factor (SCF) and endothelin-1 (ET-1) (Moretti *et al.*,2002; Moretti *et al.*, 2009). TNF-α production might contribute to keratinocyte apoptosis, resulting in reduced release of melanogenic cytokines (Moretti *et al.*, 2009). In addition, a recent study demonstrated that the TNF-α -308G/A polymorphism is common in vitiligo patients and is associated with cytokine production (Namian *et al.*, 2009).

1.4.3.5 Participation of other cells:

Abnormalities in other cell types have been observed in vitiligo. In a recent study of peripheral blood mononuclear cells of vitiligo patients, genomic and gene specific DNA methylation levels were investigated to relate the changes to the expression of genes that regulate methylation and the IL-10 gene. The results showed increased genomic DNA methylation in mononuclear cells in vitiligo patients compared with controls ($p = 0.012$). Interestingly, IL-10 exression was decreased in vitiligo patients ($p = 0.030$) and an IL-10 enhancer region was shown to be hypermethylated ($p = 0.014$), demonstrating involvement of the autoimmunity-related genes (Zhao *et al.*, 2010). Likewise, increased macrophage infiltration in perilesional skin, probably due to clearing activity, and an increase in Langerhans cells in perilesional vitiliginous skin showing degenerative changes have been reported (Le Poole *et al.*, 1996), although the participation of Langerhans cells remains unclear. Dendritic cell involvement in vitiligo is also not clear, but they might recruit T cells in the epidermis, possibly carrying out a cytotoxic function mediating the killing of stress melanocytes contributing to depigmentation (Le Poole *et al.*, 2004). In addition, alterations in natural killer (NK) cells have been demonstrated in vitiligo. Studies have found higher NK cell activity with significantly increased percent- ages of the activatory receptors, $CD16^+CD56^+$, $CD3^+CD16^+CD56^+$ and a decrease in the inhibitory receptor $CD16^+CD158a^+$ in vitiligo patients (Basak *et al.*, 2008).

1.4.3.6 Defective apoptosis and generation of autoimmunity:

Many signals that may originate either endogenously or exogenously have been shown to influence life processes. These include hormones, immune killing, ROS, genetic and physical trauma, oncogene expression, etc. Vitiligo melanocytes may have an intrinsic defect leading to melanocyte death. These melanocytes demonstrate various abnormalities, including abnormal, rough endoplasmic reticulum and incompetent synthesis and processing of melanocytes. In addition, homing-receptor dysregulation has also been detected. Early apoptosis of melanocytes has also been suggested as a cause of reduced melanocyte survival (Ortonne and Bose, 1993; van den Wijngaard *et al.*, 2000).

The execution of Programmed Cell death (PCD) is often associated with characteristic morphological and biochemical changes. Apoptotic hallmarks include membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage and fragmentation, etc. (Mignotte and Vayssiere, 1998). During apoptotic breakdown many nuclear constituents are post-translationally modified altering antigenicity. It is therefore speculated that failure to achieve PCD and clear apoptotic cell fragments may be a key pathological factor leading to autoimmune disorders. Autoimmunity could result from a failure to kill an autoreactive cell or by inducing autoimmunity against apoptotically modified cellular constituents. Therefore, the process of apoptosis may provide a source of nuclear antigens to drive the autoantibody response and provide antigens in SLE. Reports have suggested that apoptosis is abnormal in autoimmune diseases and may play a role in the induction of autoimmunity. Studies on apoptosis and clearance of apoptotic cells in lupus have shed light on the development and course of the disease. During maturation of the immune system, apoptosis of autoreactive lymphocytes in the central lymphoid organs underlies the development of tolerance. Whenever apoptotic cells accumulate due to an increased rate of apoptosis, decreased elimination or both, tolerance can be broken. Disturbances in any one of the many factors that regulate the apoptotic process might change the balance in the immune system and may predispose for the development of autoimmune phenomenon (Bijl *et al.*, 2001).

Nitric Oxide (NO) is a reactive endogenous molecule with multiple functions including inflammation and immunity. Studies have shown that nitric oxide could inhibit the *de novo* attachment of melanocytes to extra cellular matrix (ECM) suggesting that NO induced aberrant perturbation of melanocyte - ECM interaction could be a reason for melanocyte loss in vitiliginous lesions. However, Ivanova *et al.*, showed that NO induced apoptosis mediated detachment of both normal melanocytes and vitiliginous melanocytes from fibronectin occurs in a similar mechanism, suggesting that non-lesional vitiliginous melanocytes are not characterized by an increased proneness to NO induced apoptosis (Ivanova *et al.*, 2005).

A few studies suggest that loss of melanocytes in vitiligo is the result of apoptosis (Huang *et al.*, 2002). Oxidative stress, which can induce apoptosis by cytochrome c mediated pathway of caspase activation (Simon *et al.*, 2000), may contribute to

melanocyte destruction in vitiligo lesions. During apoptosis, modification of melanocytic antigens through proteolysis, changes in the phosphorylation state and citrullination may give rise to potentially immunostimulatory forms of intracellular or membrane-associated autoantigens. Generally, the efficient clearance of apoptotic cells results in the exposure of intracellular self-antigens to the immune system under non-inflammatory conditions. It has been proposed that under these conditions circulating dendritic cell precursors take up apoptotic cells and travel to lymphoid organs, where they present autoantigens from apoptotic cells to T cells in the absence of costimulatory molecules, leading to tolerance induction.

However, under the proinflammatory cytokine and extracellular acidic environment of vitiligo lesions, or the release of proteases secondary to tissue injury (Koebner reaction), these modified autoantigens, which may also expose cryptic epitopes, may be processed by mature Langerhans cells and presented to T cells. Subsequently, the autoreactive $CD4^+$ T cells may stimulate autoreactive B cells to produce autoantibodies, whereas $CD8^+$ T cells may attack melanocytes directly. It is worth noting that efficient clearance of apoptotic cells is crucial for the avoidance of autoimmune responses to intracellular antigens (Casiano and Pacheco, 2006).

1.4.3.7 Interplay of oxidative stress and immune system:

Vitiligo pathogenesis is an extremely complex event involving both genetic susceptibility as well as environmental triggers. The two major theories of vitiligo pathogenesis include an autoimmune etiology for the disease and an oxidative stress mediated toxicity in the melanocyte. Although these two theories are often presented as mutually exclusive entities, it is likely that vitiligo pathogenesis may involve both oxidative stress and autoimmune events, for which there is variability within a patient. Reactive oxygen species are recognized as important signaling molecules within the cells of the immune system. This is, at least in part, because of the reversible activation of kinases, phosphatases and transcription factors by modification of critical thiol residues (Grifiths, 2005). In fact, free radicals are involved in specific early events of T-cell activation and antioxidants reduce T-cell proliferation, IL-2R expression and IL-2 production (Chaudhri *et al.*, 1998).

Reactive oxygen species are produced as byproducts of melanogenesis in melanocytes, and controlled by several redundant antioxidant enzymes such as catalase and glutathione peroxidase, both of which are decreased in the epidermis of vitiligo patients (Schallreuter, 1999a). Oxidative stress plays a very important role in the immune system, as phagocytic cells generate reactive oxygen intermediates such as superoxide, hydrogen peroxide and nitric oxide, which are toxic to many pathogens, and at the same time they can be toxic to the host as well. Given the role of oxidative stress in both melanogenesis and in the immune system it can be hypothesized that biochemical defects in the melanin biosynthesis pathway, as well as possible defects in patient's antioxidant enzymes, are responsible for the generation of reactive oxygen species in the epidermis of vitiligo patients (Casp *et al.*, 2002). Build up of ROS along with possible immune system defects allow for the inappropriate autoimmune response against normal melanocytes.

In autoimmune disorders the immune system aberrantly targets host cells for destruction, often creating a chronic or relapsing inflammatory milieu. The effects of chronic inflammation can be devastating on the host, eventually causing damage and/or destruction of the target organ. In this inflammatory environment, ROS can accumulate with a toxic effect on surrounding cells. This can explain the pathogenesis of inflammatory vitiligo (Buckley 1953). In this rare disorder a raised rim surrounds the depigmented lesion. The question that lies unanswered is what is causing this aberrant inflammatory response in autoimmunity and whether these ROS are a result of the chronic inflammation and autoimmunity, or part of the cause of the autoimmune response.

Oxidative processes enhance the reaction of the adaptive response. Oxidation of carbohydrates enhances the antibody response to co-administered co-antigens. Moreover, the administration of the Schiff base-forming agent tucaresol during immunization with protein antigen increased T-cell-dependent immune response. Direct modification of protein antigen has been demonstrated for the enhanced immune response (Kurien *et al.*, 2006).

Oxidative stress, produced through increased catecholamine release or from other sources such as toxic intermediates of melanin precursors, can also initiate, or at least

amplify, the autoimmune destruction of melanocytes as well. Cutaneous axon terminals and epidermal melanocytes make contact via chemical synapses in human skin. An increased release of catecholamines from the autonomic nerve endings in the microenvironment of melanocytes in the vitiliginous areas has been reported. Norepinephrine is known for having direct and indirect melanocytotoxic effects. Direct actions include interacting with cellular sulfhydryl groups, enzyme inhibition, impairing mitochondrial calcium uptake, and forming some cytotoxic products including free radicals. The indirect effects include activating α-receptors of the arterioles, causing a severe vasoconstriction, and thereby producing toxic oxygen radicals caused by hypoxia (Namazi, 2003).

Hypoxia induces extracellular acidosis. It is demonstrated that extracellular acidosis activates dendritic cells. It increases the acquisition of extracellular antigens for MHC class I-restricted presentation and the ability of antigen-pulsed dendritic cells to induce both specific CD8+ cytotoxic T lymphocytes and B-cell responses (Vermeulen *et al.*, 2004). It is also demonstrated that hypoxia contributes to the initiation of adaptive immune responses by dendritic cells, favoring the development of Th1 immunity (Martı´nez *et al.*, 2007). Moreover, there are several ways by which toxic oxygen radicals, besides having a direct melanocytotoxicity, can induce an autoimmune attack against melanocytes.

The melanogenic pathway involves the formation and polymerization of L-Tyrosine. Its first and rate-limiting step is the conversion of L-tyrosine into L-dopaquinone which involves O-quinone as an intermediate product. Exposure to UV radiation for longer time causes the spontaneous production of O-quinone that further leads to the formation of H_2O_2 as a by-product (Cervantes *et al.*, 2001) (Figure 8, pathway 4).
The structures of melanocytic macromolecules and small molecules, such as Melan-A and tyrosinase, may markedly change by acute or chronic oxidative stress and can act as antigens (neo-antigens). Neo-antigens with sufficient homology or identity to host antigenic proteins induce auto-reactivity. This phenomenon is referred to as 'molecular mimicry' (Kannan, 2006). Aldehydic products, mainly the 4-hydroxy-2-alkenals, form adducts with proteins and make them highly immunogenic (Kurien *et al.*, 2006). Hydroxyl radicals are also very highly reactive and could attack a wide range of targets. The presence of rheumatoid factors in the sera and lesions of vitiligo

patients can be explained by this mechanism (Vahedi Darmian *et al.*, 2004). Over time, chronic oxidative stress could generate several adducted and / or non-adducted molecules that would essentially act as a 'neo-antigens'. This is consistent with the slow maturation of auto-antibodies in the evolution of this disease. More than one neo-antigens / autoantigens are involved in amplifying the autoaggressive lymphocytes by a process referred to as 'antigen spreading'. This is an autoimmune reaction initially directed against a single autoantigen that spreads to other autoantigens, causing the T helper cells to recognize them (Kannan, 2006).

Westerhof *et al.* (2007) suggested that phenols / catechols, which are increased in vitiliginous areas, may serve as a preferred surrogate substrate of tyrosinase, competing with tyrosine and converting into reactive quinones. Such reactive quinones, whose production is enhanced by increased hydrogen peroxide in the vitiligo lesions, can covalently bind to tyrosinase (haptenation). This could give rise to a neo-antigen, carried by Langerhans cells to the regional lymph nodes and stimulating the proliferation of cytotoxic T cells (Song, 1997).

Moreover, a few studies suggest that Melan-A is the principal vitiligo autoantigen, as high frequencies of Melan-A specific $CD8^+$ cells were found in vitiligo and seem to be correlated with the extent and severity of the disease (Lang *et al.*, 2001; Ogg *et al.*, 1998).

During chronic oxidative stress, neo-antigens potentially cause tissue damage and release a plethora of sequestered auto-antigens. This process is referred to as the 'bystander effect'. Such an outburst of auto-antigens from the target tissue would potentially amplify the effect of the neo-antigens, leading to the breakdown of self-tolerance (Kannan, 2006).

Hypoxia directly induces mitochondrial reactive oxygen species, which subsequently activate NF-κB through activation of kinases (Chandel *et al.*, 2000) (Figure 3). On activation, NF-κB regulates the expression of almost 400 different genes, which include enzymes such as iNOS, cytokines (such as TNF-α, IL-1, and chemokines), and adhesion molecules (Ahn and Aggarwal, 2005). It deserves noting that TNF-α can increase mitochondrial reactive oxygen species production through an autocrine effect

on a cell membrane receptor (Santos-Rosa *et al.*, 1999). The possible cross talk between cellular and humoral immune mechanisms in vitiligo is given in the Figure 9.

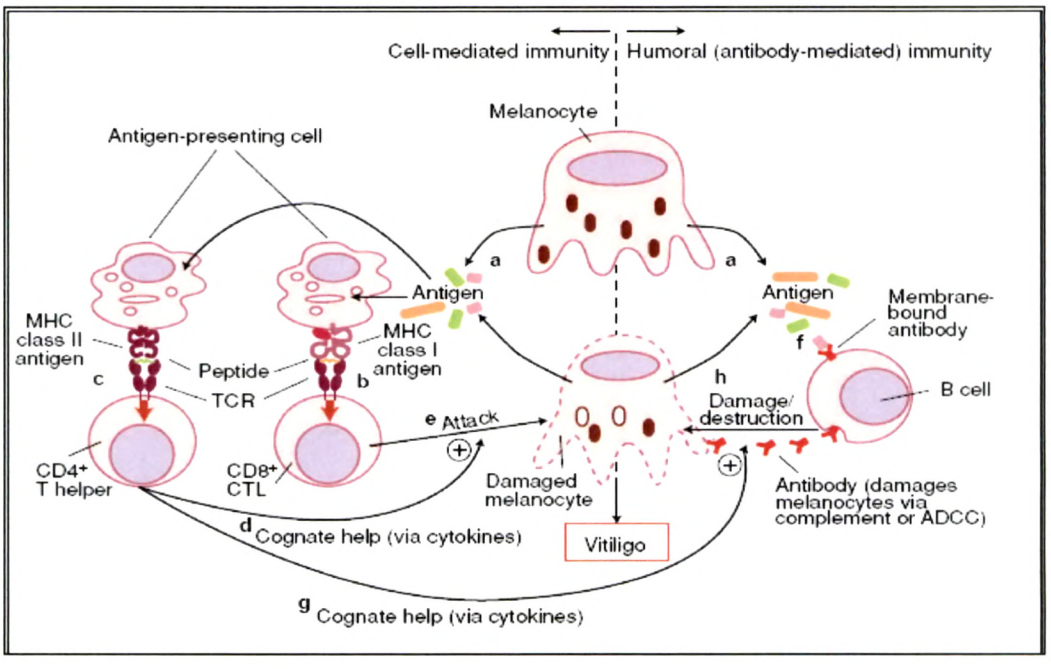

Figure 9. Possible cellular and humoral immune mechanisms in vitiligo (Kemp *et al.*, 2001).

1.4.4 Convergence Theory:

Several hypotheses on the mechanism of pathogenesis of vitiligo have been combined and formulated a convergence theory to explain the etiopathogenesis of vitiligo (Le Poole *et al* 1993). This theory states that stress, accumulation of toxic compounds, infection, autoimmunity, mutations, altered cellular environment and impaired melanocyte migration and proliferation can contribute to vitiligo pathogenesis in varying proportions.

According to the new hypothesis put forward by Dell'Anna and Picardo (Dell'Anna and Picardo 2006), a compromised membrane could render the cell sensitive to external and internal agents differentially. According to this hypothesis, the melanocytes present biochemical defects, probably due to a genetic background, affecting the structure and functionality of the membranes. A compromised

membrane could render the cell sensitive to external and internal agents differently (UV, cytokines, catechols, melanin intermediates, growth factor withdrawal) usually ineffective on cell activity and survival. The impaired arrangement of the lipids, involving fatty acids and cholesterol, may affect the transmembrane housing of proteins with enzymatic or receptor activities. The altered expression and release of transmembrane proteins could be basis for the exposure of 'new antigens' triggering an immune response (Broquet *et al* 2003; Kroll *et al* 2005). The final result could depend on the intensity or duration of the stimuli; a mild aggression leading to a reduction of ATP production impairs the adhesion function; a great stimulus acting as pro apoptotic agent affects mitochondrial cell survival check points; finally, a strong stress directly causing the necrotic death with an inflammatory, or at least lymphocytic infiltrate (Dell'Anna and Picardo 2006).

1.5 VITILIGO GENETICS:

Vitiligo is characterized by multiple susceptibility loci, incomplete penetrance, and genetic heterogeneity (Spritz *et al.*, 2008). The inheritance of vitiligo may involve genes associated with the biosynthesis of melanin, response to oxidative stress and regulation of autoimmunity. It is however not yet clear as to whether the abnormalities observed in neural pathways, oxidative stress and autoimmune events represent a cause or effect response of the disease. Nevertheless, it may involve both genetic and environmental factors. Recent studies suggest that genetic factors may play a major role in the pathogenesis of vitiligo. There is a positive family history in about 20% of cases and similar concordance in identical twins (Spritz, 2008). Shajil *et al* (2006) also reported that 21.93% of Gujarat vitiligo patients exhibited positive family history and 13.68% patients had at least one affected first-degree relative.

Vitiligo is a polygenic disease, and attempts have been made to identify genes involved in susceptibility include gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses to discover new genes. Recent genome-wide association studies (GWAS), have identified generalized vitiligo susceptibility genes which involve immune regulation and immune targeting of melanocytes, suggesting that generalized vitiligo is a primary autoimmune disease

however, the biological triggers of the autoimmune process remain unknown (Spritz *et al.*, 2011).

Considerable progress has been made in the identification of candidate genes. Such studies have been carried out in different populations with differing susceptibility factors. Systematic studies in different populations would help identify candidate genes governing oxidative stress that contribute to the pathogenesis of vitiligo. As immune system also seems to play a major role in vitiligo, understanding the mechanism of immune responses involved and, identification of the potential antigen/s in a particular population would help in designing therapeutic regimens by neutralizing the specific cell types or masking the specific antigen/s that are involved in the pathogenesis of vitiligo. The mammalian skin pigmentation is possibly controlled by more than a hundred genes; these genes as well as the genes regulating oxidative stress and immune responses qualify as potential candidate genes for vitiligo.

The role of genetic factors in vitiligo was also considered early because of the frequent clustering of cases among close relatives (Stuttgen, 1950; Teindel, 1950), and eventual genetic epidemiological studies by Das *et al.* (1985) supported multifactorial, polygenic inheritance, which currently is termed ''complex disease''. In 1960s and 1970s, ABO, haptoglobin, erythrocyte enzymes, and various serum proteins were tested as genetic markers for vitiligo, with negative results. In the 1970s, a plethora of analyses of HLA in vitiligo were reported, with equivocal and conflicting findings. Some of the earliest genetic studies of vitiligo were carried out in India, of ABO blood groups (Kareemullah et al., 1977), α1-antitrypsin, and haptoglobin (Mujahid et al., 1990). Several candidate genes have been tested for genetic association with generalized vitiligo, including the *MHC, ACE, CAT, CTLA4, COMT, ESR, GCH1, MBL2, PTPN22,* and *VDR* (Spritz 2007, 2008). Most of these studies reported significant associations, although some yielded only marginal significance and several were not replicated by subsequent studies. Recently, a number of genes which play a role in vitiligo susceptibility, including *HLA, NALP1, XBP1, FOXP1, IL2RA* have been tested for genetic association with vitiligo (Spritz, 2010).

HLA molecules present peptides to T-cells, and it has been proposed that certain HLA haplotypes confer more efficient presentation of cognate autoantigen, thereby predisposing to autoimmunity; an example is HLADQB1*0301 (Gilhar *et al.,* 2007). *NALP1* is involved in the innate immune response to pathogens. Recent fine-mapping studies showed associations with chromosomes 7 and 9 (Jin *et al.,* 2009). Numerous other candidate genes and susceptibility loci bear ongoing scrutiny, including *CAT*, *GST*, *COMT*, *ACE*, mannose-binding lectin 2 and *XBP1* (Casp *et al.*,2002;Onay *et al.*, 2007; Liu *et al.,* 2009; Ren *et al.,* 2009). A recent genome-wide association study by Jin *et al.* (2010) using European white subjects and controls showed significant associations of generalized vitiligo with the following loci, which have been previously linked with autoimmune diseases: HLA class I and II molecules, *PTPN22*, *LPP*, *IL2RA*, *UBASH3A* and *C1QTNF6*. Two additional immune-related loci identified were *RERE* and *GZMB*. The HLA class I association occurred in the regions between *HLA-A* and *HCG9*, consistent with previous reports of strong associations with the HLA-A*02 allele, and the HLA class II gene association occurred in the region between *HLADRB1* and *HLADQA1*, in keeping with known associations to the HLA-DRB1*04 allele. With the exception of *PTPN22*, the associations were similar whether patients had vitiligo alone or vitiligo as well as another autoimmune disease. An important association with a non-immune related gene, tyrosinase was identified. Tyrosinase is a melanocyte enzyme that catalyzes the rate limiting step in melanin biosynthesis and is a putative target autoantigen in vitiligo. Interestingly, certain *TYR* SNPs are associated with melanoma risk, and some of these are in linkage disequilibrium with vitiligo. Vitiligo *TYR* SNPs could be more antigenic than melanoma *TYR* SNPs, thereby conferring protection from melanoma through immune surveillance (Jin *et al.*, 2010; Bishop *et al.*, 2009).

1.5.1 SINGLE NUCLEOTIDE POLYMORPHISMS

Humans are 99.9% genetically identical (Venter *et al.,* 2001) and the most common type of genetic variability found in humans is in the form of Single Nucleotide Polymorphisms (SNPs). A SNP refers to a single base change in DNA. These SNPs occur when there are two or more possible nucleotides are seen at a specific mapped location in the genome, where in the least frequent allele has an abundance of 1% or

more (Brookes *et al.,* 1999). An International Single Nucleotide Polymorphism Consortium (ISNPC) has currently identified over 6 million SNPs, approximately one at every 1-2 kilobase. SNPs may occur in non-coding regions as well as in coding regions. Some missense polymorphisms are more conservative than others e.g. a change in the codon CUU (leucine) to AUU (isoleucine) would have minimal structural impact, whereas modification of CAU (histidine) to CCU (proline) would be expected to have dramatic structural and/or functional influence on the protein.

SNPs act as potential useful markers for the gene mapping studies, particularly for identifying genes involved in complex diseases (Chakravarti *et al.*, 2001). But the knowledge of frequency and distribution of these SNPs across ethnically diverse populations is essential in order to know their usefulness as markers for gene mapping studies. Additionally, the density of SNPs needed for mapping complex diseases will likely vary across populations with distinct demographic histories (Tishkoff and Verrelli 2003).

The Common Disease/ Common Variant hypothesis states that common genetic disorders are affected by common disease susceptibility alleles at a few loci that are at high frequency across ethnically diverse populations e.g. the APOE ε 4 allele is associated with increased risk for Alzheimer's disease (Chakravarti *et al.,* 1999; Goldstein and Chikhi 2002). Thus, these alleles might arise prior to population differentiation. Alternatively, some complex diseases may be influenced by rare susceptibility alleles at many loci. If these disease predisposing alleles are geographically distributed due to mutation, drift, or regional specific selection pressure, then characterizing SNP diversity, haplotype structure and linkage disequilibrium across a broad range of ethnically diverse populations is of particular importance for identifying disease predisposing alleles (Tishkoff and Williams 2002).

1.5.1.1 SNP analysis:

SNP analysis techniques fall into two distinct classes:

I. SNP Identification: Detection of novel polymorphisms

II. SNP Genotyping: Identifying specific allele in a known population.

1.5.1.1.1 SNP identification methods:

The identification and characterization of large numbers of SNPs are necessary before their use as genetic tools. The following four methods are commonly used for SNP detection (Gray *et al.*, 2000).

1.5.1.1.1.1 SSCP detection:

For single strand conformation polymorphism (SSCP) detection, the DNA fragment spanning the putative SNP is PCR amplified, denatured and run on denaturing polyacrylamide gel. During the gel run, the single-stranded fragments adopt secondary structures according to their sequences. Fragments bearing SNPs are identified by their aberrant migration pattern and are further confirmed by sequencing. Although SSCP is a widely used and relatively simple technique, it gives a variable success rate for SNP detection, typically ranging from 70 to 95%. It is labor intensive and has relatively low throughput, although higher capacity methods are under development using capillary-rather than gel based detection (Orita *et al.,* 1989).

1.5.1.1.1.2 Heteroduplex analysis:

This relies on the detection of a heteroduplex formed during reannealing of the denatured strands of a PCR product derived from an individual heterozygous for the SNP. The heteroduplex can be detected as a band shift on a gel, or by differential retention on a HPLC column. HPLC has rapidly become a popular method for heteroduplex-based SNP detection due to simplicity, low cost and high rate of detection i.e. 95-100% (Lichten and Fox 1983).

1.5.1.1.1.3 Direct DNA sequencing:

The favored high-throughput method for SNP detection is direct DNA sequencing. SNPs may be detected *in silico* at the DNA sequence level. The wealth of redundant sequence data deposited in public databases in recent years, in particular expressed sequence tag (EST) sequences, allows SNPs to be detected by comparing multiple versions of the same sequence from different sources.

1.5.1.1.1.4 Variant detector arrays (VDA):

VDA technology is a relatively recent addition to the high throughput tools available for SNP detection. This technique allows the identification of SNPs by hybridization

of a PCR product to oligonucleotides arrayed on a glass chip and measuring the difference in hybridization strength between matched and mismatched oligonucleotides. The VDA detection allows rapid scanning of large amounts of DNA sequences (Wang *et al.,* 1998).

1.5.1.1.1.5 High Resolution Melting (HRM):

High Resolution Melting (HRM) is a novel, homogeneous, close-tube, post-PCR method, enabling analysis of genetic variations (SNPs, mutations, methylations) in PCR amplicons. HRM characterizes nucleic acid samples based on their disassociation (melting) behavior. Samples can be discriminated according to their sequence, length, GC content or strand complementarity. Even single base changes such as SNPs (single nucleotide polymorphisms) can be readily identified (Reed *et al.*, 2004).

1.5.1.1.2 SNP GENOTYPING METHODS:

SNP genotyping involves two components (Chen and Sullivan 2003) i.e. a method for discrimination between alternate alleles and a method for reporting the presence of the allele or alleles in the given DNA sample.

A typical genotyping protocol consists of the following steps.

1. Target fragment amplification by PCR.
2. Allelic discrimination reaction can be carried out by either of the following methods: primer extension, pyrosequencing, hybridization and sequence specific cleavage.
3. Allele specific product identification can be done by either of the following ways. Fluorescence resonance energy transfer (FRET), electrophoresis, microarray and mass spectroscopy.
4. Taqman assay for SNP genotyping: The TaqMan genotyping assay combines hybridization and 5' nuclease activity of polymerase coupled with fluorescence detection. It allows screening, association, candidate region, candidate gene, and finemapping studies

1.5.1.2 Association studies:

SNP based studies can be performed mainly for two purposes:

a. Direct testing of a SNP with functional consequence for association with a disease trait.
b. Using a SNP as a marker for linkage disequilibrium.

1.6 Candiate genes associated with vitiligo susceptibility:

The complex genetics of vitiligo involves multiple susceptibility loci, genetic heterogeneity and incomplete penetrance with gene-gene and gene-environment interactions (Zhang, 2005). A few genes that are identified to contribute to vitiligo susceptibility are given in the Table 2.

Table 2. Genes that contribute to vitiligo susceptibility.

Gene	Method	SNP	Reference
ACE	*Candidate gene association*	rs1799752	Jin *et al.*, 2004a
AIRE	*Candidate gene association*	rs1800521	Nagamine *et al.*,1997
ASIP	*Candidate gene association*	rs6058017	Na *et al.*, 2003
CAT	*Candidate gene association*	rs769217 rs7943316	Casp *et al.* 2002, Gavalas *et al.* 2006, Park *et al.* 2006.
CD4	*Candidate gene association*	CD4 pentanucleotide repeat	Zamani *et al.*,2009 Kristiansen et al., 2004
CLEC11A	*Candidate gene association*	rs7246355	Lan *et al.*,2009
COMT	*Candidate gene association*	rs4680	Tursen *et al.*,2002
CTLA4	*Candidate gene association*	rs231775	Kemp *et al.*, 1999
C12orf10	*Candidate gene association*	rs7975232	Philips *et al.*,2010
DDR1			
EDN1	*Candidate gene association*	rs2267641	de Castro *et al.*,2010
		rs2071942-	Kim *et al.*,2007

	Candidate gene association	rs5370	Jin *et al.*, 2004b
ESR1			Li *et al.*,2008
FAS	*Candidate gene association*	rs2234693	Li M *et al.*,2009
	Candidate gene association	rs2234767	Alkhateeb et al., 2005
FOXD3		rs41285370	Hori *et al.*,2003
FOXP3	*Genome-wide linkage*	rs11798415	Uhm *et al.*,2007
	Defective in IPEX syndrome	rs2071487	
GSTM1			Liu *et al.*,2009
GSTT1	*Candidate gene association*	rs2234953	
IL1RN	*Candidate gene association*	IL1RN VNTR	Pehlivan *et al.*,2009
IL10	*Candidate gene association*	rs689466	Abanmi *et al.*,2008
	Candidate gene association	rs1800872; rs1800871	
KITLG		rs11104947	Lan *et al.*,2009
MC1R	*Candidate gene association*	rs2228479	Na *et al.*, 2003
MBL2	*Candidate gene association*	rs6721961	Onay *et al.*,2007
NFE2L2	*Candidate gene association*	rs36901	Guan *et al.*,2008
NALP1	*Candidate gene association*	rs6502867	Jin *et al.*, 2007
PDGFRA-KIT	*Candidate gene association*	rs689466	Chen *et al.*, 2005
PTPN22	*Candidate gene association*	rs2476601	Canton *et al.*, 2005
PTGS2	*DNA sequencing*	rs7574865	Li K *et al.*,2009
STAT4	*Candidate gene association*	rs1135216	Hu *et al.*,2010
TAP1	*Candidate gene association*	rs2005061	Casp *et al.*,2003
TGFBR2	*Candidate gene association*	rs1800629	Yun *et al.*,2010
TNF	*Candidate gene association*	rs3806933	D'Alfonso *et l.*,1994
			Pociot *et al.*, 1993

TSLP	*Candidate gene association*	rs1043784	
TXNDC5	*Candidate gene association*	rs1458836-	Birlea *et al.*,2011
UVRAG	*Candidate gene association*	rs7933235	Jeong *et al.*,2010a
VDR	*Candidate gene association*	rs7975232	Jeong *et al.*,2010b
XBP	*Candidate gene association*	rs2269577	Birlea *et al.*,2006
	Candidate gene association	rs2269577	Birlea *et al.*,2011

1.6.1 *AIRE*

Vitiligo is commonly associated with autoimmune polyglandular syndrome type I (APS I) (Ahonen *et al.*,1990) and mutation in *AIRE* gene causes this disease. *AIRE* gene is normally expressed in immune related organs such as thymus and lymph nodes. The function of AIRE protein is to act as a transcription factor (Nagamine *et al.*, 1997). Mutation analysis has identified two mutations in this gene in Swiss and Finnish APS I patients (Nagamine *et al.*, 1997).

1.6.2 *CAT*

Catalase converts hydrogen peroxide to water and thereby prevents the cell damage from highly reactive oxygen derived radicals. The *CAT* gene is considered as a candidate gene because of the reduction in catalase activity and concomitant accumulation of H_2O_2 is observed in the epidermis of vitiligo patients (Schallreuter *et al.*, 1991). An association has been established between vitiligo and a SNP in exon 9 of *CAT* gene (Casp *et al.*, 2002; Gavalas *et al.*, 2004). It has been reported that C/T heterozygotes are more frequent among vitiligo patients than controls. The C allele is transmitted more frequently to patients than controls, which suggests that linked mutations in or near the *CAT* gene may contribute to a quantitative deficiency of catalase activity in vitiligo patients and the accumulation of H_2O_2.

1.6.3 *COMT*

In melanocytes, COMT prevents the formation of toxic o-quinones during melanin synthesis (Pavel *et al.*, 1983). It was found that epidermal homogenates from vitiligo patients expressed altered levels of COMT activity than homogenates from healthy controls (Le Poole *et al.*, 1994). A common biallelic polymorphism in the *COMT*

gene that determines high and low enzyme activity has been associated with neuropsychiatric disorders (Karayiorgou *et al.,* 1997). *COMT* polymorphism has not been detected in vitiligo patients compared to controls. However, *COMT*- LL (low activity homozygote) genotype was found to be significantly associated with acrofacial vitiligo (Tursen *et al.,* 2002).

1.6.4 *LMP* and *TAP*

Genes within the class II region of the major histocompatibility complex (MHC) are reported to be associated with several autoimmune diseases (Tanaka *et al.,* 1998; Pamer and Cresswell 1998). This highly polymorphic region includes several genes involved in the processing and presentation of antigens to the immune system including low molecular weight protein polypeptide 2 and 7 (LMP 2 and 7) and transporter associated with antigen processing protein 1 (TAP 1). Casp *et al.,* (2003) showed genetic association of early onset of vitiligo with the *TAP1* gene. Moreover alleles from heterozygous parents were disequilibratedly transmitted to affected offspring for the *TAP1* gene, as well as for the closely linked *LMP2* and *LMP7* genes (Casp *et al.,* 2003).

1.6.5 *MC1R* and *ASIP*

Polymorphism studies in *MCIR* and *ASIP* revealed that G274A and A488G represented abundant forms of the SNPs of the *MCIR* in Korean population. The frequency of the A allele of G274A was higher in vitiligo patients; however this SNP was not statistically significant.The patients who carried both the SNPs of *MCIR* and *ASIP* were prone to vitiligo (Na *et al.,* 2003).

1.6.6 ESR1

It was reported that high estrogen levels in the serum was associated with increased skin pigmentation (Shahrad and Marks 1977). Successful treatment of vitiligo was shown with the steroid- thyroid hormone mixture containing estrogen (Nagai *et al.,* 2000; Ichimiya *et al.,* 1999). It was shown that *ESRI* (Estrogen receptor gene 1) intron 1 C/T polymorphism was associated with female or generalized vitiligo patients.

ESR1 gene may be a possible risk factor for the female or generalized type of vitiligo (Jin *et al.*, 2004b).

1.6.7 *KIT*

KIT encodes for a tyrosine kinase receptor named c-kit expressed on the surface of melanocytes, mast cells, germ cells and hematopoietic stem cells (Grabbe *et al.*, 1994). The c-kit ligand, SCF (stem cell factor) is involved in the proliferation and survival of melanoblasts and may be associated with the dysfunction and/or loss of melanocytes (Nishikawa *et al.*, 1991). The expression of c-kit and its down stream effector microphthalmia associated transcription factor (MITF) is reduced in vitiligo epidermis (Norris *et al.*, 1996; Kitamura *et al.*, 2004). It was also observed that vitiligo had remained stable for many years after treatment with tyrosine kinase inhibitors (Passeron and Ortonne 2005). Moreover, several cases of vitiliginous depigmentation occurring after treatment with new tyrosine kinase inhibitors (STI-571 and SU 11428) are reported (Raanani *et al.*, 2002). BCL2 is a MITF dependent KIT transcriptional target in melanocytes (McGill *et al.*, 2002) and a decrease in *BCL 2* expression in melanocytes increases their susceptibility to apoptosis. Interestingly, SCF strongly protects melanocytes form TNF related apoptosis inducing ligand (TRAIL) (Larribere *et al.*, 2004). SCF/c – KIT thus brings new interesting potential clues regarding the physio-pathology of vitiligo.

1.6.8 *FOXD3*

Forkhead box D3 (*FOXD3*) is a transcription factor that suppresses melanoblast development from neural crest (Kos *et al.*, 2001). Therefore dysregulated expression might harm melanocytes. FOXD3 also regulates endodermal differentiation including thyroid, pancreas, adrenal gland and gut (Guo *et al.*, 2002). Also other FOX factors are involved in autoimmune syndromes (Jonsson and Peng 2005). Mutations in *FOXD3* leading to elevated FOXD3 transcription is recently reported in one AIS 1 linked family (Alkhateeb *et al.*, 2005).

1.6.9 *TNFA*

TNFA expression may depend on polymorphisms in the *TNFA* promoter region or a linkage association with the HLA genotype (D'Alfonso *et al.,* 1994; Pociot *et al.,* 1993). In this respect, the -308 allele is associated with the HLA A1, B8, DR3, DR4 and the DQ2 haplotypes; the DR2 is associated with low TNFα responses; and the DR3 and DR4 genotypes are associated with high TNFα production (Wilson *et al.,* 1997; Yucesoy *et al.,* 2001). The increased HLA associations in vitiligo patients were: HLA-A2, A31, B13, B27, B56, B60, CW6, DR4, DR5, DR7, DR53 and DQ3 (Zhang *et al.,* 2005). Therefore, the increased production of TNFα could contribute to the increased incidence of vitiligo observed in individuals with DR4 haplotype. However, a study in the Turkish population shows that -308 *TNFα* promoter polymorphism has no significant influence on vitiligo susceptibility and clinical manifestations (Yazici *et al.,* 2006).

1.6.10 *CD4*

The *CD4* gene plays an important role in the cell-mediated immune response and its association with type 1 diabetes mellitus has been previously reported. Zamani *et al.,* (2009) reported an association with a pentanucleotide variable number of tandem repeats polymorphism (VNTR) with vitiligo.

1.6.11 *CLEC11A*

C-type Lectin Domain Family 11, Member A *(CLEC11A)* is one of the relevant keratinocyte-growth related genes and its role has been implicated in the pathogenesis of vitiligo vulgaris (Lan *et al.,* 2009).

1.6.12 *DDR1* (Discoidin domain receptor tyrosine kinase 1)

Receptor tyrosine kinases play a key role in the communication of cells with their microenvironment. These kinases are involved in the regulation of cell growth, differentiation and metabolism. The protein encoded by this gene belongs to a subfamily of tyrosine kinase receptors. Sakuma *et al.* (1996) cloned genomic DNA of the *DDR1* gene. The gene contains 15 exons spanning approximately 9 kb. The

promoter region of the gene contains a consensus binding site for p53. Silva de Castro *et al.* (2010), reported association of *DDR1* gene with vitiligo. However, Kim *et al.* (2010) found no association of vitiligo with *DDR1*.

1.6.13 *EDN1* (Endothelin-1)

Endothelin-1, which is expressed by keratinocytes, has paracrine effects on melanocytes, influencing their homeostasis, proliferation and pigmentation. It is thought to play a role in the skin response to 311-nm, narrow-band ultraviolet irradiation. Interestingly, the haplotype frequencies of *EDN1* polymorphisms differed significantly between vitiligo patients and healthy controls. When analysed according to clinical type, the haplotype frequencies in the focal and segmental clinical types differed significantly from healthy controls (Kim *et al.*, 2007)

1.6.14 *FAS*

The FAS/FASLG system plays a key role in regulating apoptosis. Previous findings have shown that CD4-dependent destruction of melanocytes is partially inhibited by blocking FAS-FASLG interactions in autoimmune vitiligo. Functional polymorphisms of the FAS and FASLG genes can alter their transcriptional activities. Li *et al.* (2008) and Li *et al.* (2009) reported an association of vitiligo with the *FAS* gene.

1.6.15 *FBXO11-MSH6*

The involvement of FBXO11 (previously, VIT1) in vitiligo was suggested on the basis of differential expression analysis (Le Poole *et al.*, 2001). Putative mutations in the adjacent MSH6 gene were reported in a single patient with early-onset colorectal cancer, systemic lupus erythematosus, and vitiligo (Rahner *et al.*, 2008).

1.6.16 *FOXP3*

FOXP3, a member of the fork-winged helix family of transcription factors, plays an important role in the development and function of naturally occurring CD4 (186940)-positive/CD25 (IL2RA; 147730)-positive T regulatory cells (Tregs). Tregs are

involved in active suppression of inappropriate immune responses. Due to similarities between the autoimmunity and inflammation produced by manipulation of CD25-positive/CD4-positive regulatory T (Tr, or Treg) cells and those induced by genetic defects in the *FOXP3* gene, Hori *et al.* (2003) investigated the contribution of Foxp3 to the development and/or function of Tr cells in mice. *FOXP3* is the defective gene in the X-linked recessive immunodysregulation, polyendocrinopathy, and enteropathy multiple autoimmune disease syndrome and vitiligo.

1.6.17 *GSTM* and *GSTT*1

The glutathione S-transferases (GSTs) are a family of enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens (Mannervik, 1985). Uhm *et al.* (2007) reported an association with a *GSTM1* INDEL polymorphism with vitiligo. Liu *et al.* (2009) reported an association with a *GSTT1* INDEL polymorphism with vitiligo however, another study showed no association of the same marker with vitiligo (Uhm *et al.*, 2007).

1.6.18 *IL1RN* (IL1 receptor antagonist)

IL1RN is a protein that binds to IL1 receptors (IL1R1) and inhibits the binding of IL1-alpha and IL1-beta (IL1B). As a consequence, the biological activity of these two cytokines is neutralized in physiological and pathophysiological immune and inflammatory responses. IL1RN was the first-to-be described, naturally occurring cytokine or hormone-like molecule to function as a specific receptor antagonist (Arend, 1991). Pehlivan *et al.* (2009) reported an association with an *IL1RN* intronic VNTR polymorphism, however, this result was rendered not significant by appropriate multiple-testing correction.

1.6.19 *IL10* (Interleukin 10)

IL10 family of cytokines produced by activated macrophages that targets various leukocytes and mainly represses excessive inflammatory responses. This family of cytokines also plays a role in epithelial cell proliferation, anti-apoptotic responses, tissue remodeling and healing. As IL 10 acts as an anti- inflammatory cytokine and inhibits the production of several cytokines such as IFN- γ from Th1 cells, the low

levels of this cytokine is been associated with autoimmune diseases. Abanmi *et al.* (2008) reported association of vitiligo with *IL10*.

1.6.20 *PDGFRA-Kit* (Platelet-derived growth factor receptor alpha)

The *PDGFRA* gene is a proto-oncogene that maps to 4q12. It belongs to the human type III family of transmembrane receptors, with an intrinsic tyrosine kinase component. The PDGFRA protein has been shown to be important for several cellular and tissue processes, such as proliferation, apoptosis, chemotaxis, melanogenesis, hematopoiesis and gametogenesis (Mol *et al.*, 2003). Several reports have documented PDGFRA in regulation of pigmentation. PDGF has been shown to be important for the differentiation and survival of melanocytes during embryonic development (Adamyeko *et al.*, 2009). These reports lead us to speculate that the *PDGFRA* gene may be a candidate susceptibility gene of vitiligo mapped to the region of 4q12.

1.6.21 *TSLP* (Thymic stromal lymphopoietin)

TSLP induces naïve CD4+ T cells to produce Th2 cytokines. In addition, to low production of Th2 cytokines, strong Th1 response, which plays an important role in vitiligo development, has been induced by blockade of TSLP or TSLP receptor.Cheong *et al.* (2009) reported an association with the TSLP 50-flanking SNP rs3806933 (-847C4T).

1.6.22 *UVRAG* (UV radiation resistance-associated gene)

Teitz *et al.* (1990) identified a cDNA that partially complements the ultraviolet (UV) sensitivity of xeroderma pigmentosum complementation group C (278720) cells. Perelman *et al.* (1997) named this gene *UVRAG* and reported that the 4.0 kb *UVRAG* mRNA encodes a predicted 648-amino acid protein. Jeong *et al.* (2010b) reported association with a haplotype defined by SNPs rs7933235 and rs1458836 in vitiligo.

1.6.23 *VDR* (Vitamin D receptor)

VDR is an intracellular hormone receptor that specifically binds 1,25 (OH)2D3 and mediates its effects. In 31 GV cases and 33 controls, Birlea *et al.* (2006) reported an association with the VDR restricted fragment length polymorphism rs7975232.

1.6.24 *XBP1* (X box-binding protein 1)

XBP1, is a protein which in humans is encoded by the XBP1 gene.(Liou et al.,1990) The XBP1 gene is located on chromosome 22 .The XBP1 protein is a transcription factor that regulates the expression of genes important to the proper functioning of the immune system and in the cellular stress response (Yoshida *et al.*, 2006).Spritz *et al.* (2004) detected linkage of GV to microsatellites at 22q11–q11.22 and Liang *et al.* (2007) at 22q12 in Chinese families. Ren *et al.* (2009) tested XBP1 as a positional/biological candidate gene within the linkage interval.

1.7 LINKAGE AND ASSOCIATION STUDIES:

Familial clustering and linkage disequilibrium studies showed that genetic factors predispose vitiligo although a clear transmission pattern and cosegregation of vitiligo with specific mutations have not been demonstrated.

1.7.1 HLA associations:

The frequent association of vitiligo with other autoimmune diseases has prompted the studies of HLA association with vitiligo predisposition. The *HLA* loci are strongly linked to other loci in the major histocompatibility region of chromosome 6p. Therefore, it may be that vitiligo associated *HLA* alleles are not disease causing but are genetic markers that are usually co inherited in the population (i.e. in strong linkage disequilibrium) with the actual disease allele at another locus within the major histocompatibility region (Zhang et al 2005). Linkage disequilibrium studies in different populations have consistently showed a significant association between the HLA system and vitiligo predisposition. There are several studies on the association between vitiligo and HLA complex. HLA subtypes vary with racial/ethnic background.

Association of *MHC* alleles with a disease gains importance because of the antigen-presenting function of the MHC. Recent genome wide association studies have implicated the role of MHC in vitiligo (Jin *et al.*, 2010, Quan *et al.*, 2010) where several SNPs in the *MHC* region were significantly associated with the disease. However, the authors imputed the Human Leukocyte Antigen (HLA) class-I alleles based on the linkage disequilibrium (LD) of HLA alleles with the specific SNPs, but could not impute HLA class-II alleles due to limitations of HLA allele imputation in the CHB (Chinese Han from Beijing, China) samples (Quan *et al.*, 2010). The association of multiple HLA class I and class II antigens have been suggested for vitiligo (de Vijlder et al., 2004, Zhang *et al.*, 2004a, Zhang *et al.*, 2004b) although no consensus could be reached, due to distinct ethnic groups (Orozco-Topete *et al.*, 2005, Tastan *et al.*, 2004, Zamani *et al.*, 2001) small sample sizes and low resolution typing methods used to identify the HLA antigens. For example, HLA-DR4 is increased in blacks, HLA-B13 in Moroccan Jews, and HLA-B35 in Yemenite Jews. An association of HLA-B13 with anti-thyroid antibodies has been reported (Rezaei *et al.*, 2007). However, using high resolution typing methods and large number of samples, Singh *et al.*, 2012 suggested a consistent increase of A*33:01, B*44:03, and DRB1*07:01 in both initial and replication studies implicating these alleles as possible markers of vitiligo in North India and Gujarat. These data apparently suggest auto reactive $CD4^+$ T-helper cells to be restricted by HLA-DRB1*07:01 and the auto reactive $CD8^+$ cytotoxic T cells by HLA-A*33:01, A*02:01, B*44:03, and B*57:01 in the Indian population. Previous studies in Caucasians showed association of generalized vitiligo with both MHC class I (specifically, HLA-A*02:01) (Jin *et al.*, 2010; Jin *et al.*, 2012) and class II loci (Jin *et al.*, 2010), whereas studies carried out in Chinese show no apparent association in the class I or II regions and instead favor association in the class III region (Quan *et al.*, 2010). Together, these findings indicate that the principal MHC genetic associations with generalized vitiligo differ among different populations, and may in part mediate differing prevalence of this autoimmune disease in different groups around the world. The HLA association studies reported till now are listed in Table 3.

Table 3. HLA associations reported in vitiligo.

1.7.1.1 Positive association	Negative association	Reference
HLA-A*33:01, HLA-A*02:01, HLA-B*44:03, HLA-DRB1*07:01	DRB1*03:01	Singh *et al.*, 2012
HLA-A*02:01	-	Jin *et al.*, 2012
DRB1*07:01	-	Ren *et al.*, 2009
HLA-A2	-	Liu *et al.*, 2007
DRB1*04-DQB1*0301	DRB1*15-DQB1*0602	Fain *et al.*, 2006
DQA1*0302,*0601, DQB1*0303, *0503	*0503 DQA1*0501	Yang *et al.*, 2005
A*2501, A*30, B*13, B*27, Cw*0602	A*66	Zhang *et al.*, 2004
DR4, DR53	DR3	de Vijlder *et al.*, 2004
DR3, DR4, DR7	-	Tastan *et al.*, 2004
DRB4*0101, DQB1*0303	-	Zamani *et al.*, 2001
DRB1*0701, DQB1*0201, DPB1*1601	-	Buc *et al.*, 1998
A2, A10, A30 + A31, B13, B15	A28, B46	Wang *et al.*, 2000
A2, Dw7	-	Buc *et al.*, 1996
B21, Cw6, DR53	A19, DR52	Al-Fouzan *et al.*, 1995
DR6	DQ2	Valsecchi *et al.*, 1995
Bw6, DR7	-	Venkataram *et al.*, 1995
DR6	Cw7	Venneker *et al.*, 1993
B46, A31, Cw4	-	Ando *et al.*, 1993
DR12, A2	-	Schallreuter *et al.*, 1993
A30, Cw6, DQ3	C4AQ0	Orecchia *et al.*, 1992
DR1	-	Poloy *et al.*, 1991

A30, Cw6, B27, DR7	DR1, DR3	Finco *et al.*, 1991
A2, A3	-	Dai *et al.*, 1990
DR4, DQ3	-	Dunston and Halder,1990
DR4	-	Foley *et al.*, 1983
BW35	-	Metzker *et al.*, 1980
A1, A2, A31	A10	Kachru *et al.*, 1978
Cw* 0602	-	Xia *et al.*, 2006

1.8 GENOME WIDE ASSOCIATION STUDIES (GWAS)

Genome wide linkage scans involve the typing of families using polymorphic markers that are positioned across the whole genome, followed by calculating the degree of linkage of the marker to a disease trait. Positional candidate genes can be identified by examining the regions around the peaks of linkage that are obtained by the study. Several genome wide linkage analyses of vitiligo have been performed and multiple linkages to vitiligo have been identified (Nath *et al.*, 2001; Fain *et al.*, 2003; Alkhateeb *et al.*, 2002; Spritz *et al.*, 2004). The susceptibility loci identified by genome wide linkage analyses are given in Table 4.

The most important recent vitiligo developments were two large-scale genomewide association studies of generalized vitiligo, one in Caucasians (Jin *et al.*, 2010) and the other in Chinese (Quan *et al.*, 2010), which together identified and confirmed at least 16 different loci that contribute to generalized vitiligo susceptibility. Jin *et al.*, (2010 a,b) carried out a GWAS of GV in non-Hispanic white subjects, identifying and confirming different loci that contribute to GV risk, including *FOXP1, MYH15, CCR6, ICA1, TBC1D2, IKZF4, SH2B3* almost all of which have immunoregulatory functions (Jin *et al.*, 2010a, b). Two of these loci and one additional signal in the MHC were also identified in a Chinese GWAS of GV (Quan *et al.*, 2010).

All but one of these genes encode proteins involved in regulation of the immune system and/or have been genetically associated with susceptibility to other autoimmune diseases. The sole exception is *TYR*, encoding tyrosinase, the key enzyme of melanin biosynthesis and the principal vitiligo autoimmune antigen. In

Caucasians, a common *TYR* missense variant, R402Q, confers both relative protection from generalized vitiligo and relative susceptibility to malignant melanoma, by modulating the presentation of the TYR peptide by HLA-A2*01, thereby modulating recognition of melanocytes by the immune system. These genes together account for a relatively small fraction of the genetic risk of generalized vitiligo, indicating that many additional vitiligo susceptibility genes undoubtedly remain to be discovered.

Recently, Jin *et al.*, 2012 reported a large GWAS (450 individuals with vitiligo and 3,182 controls), an independent replication study (1,440 cases and 11,316 controls) and a meta-analysis (3,187 cases and 6,723 controls) identifying 13 additional vitiligo-associated loci. These include *OCA2-HERC2, MC1R*, a region near *TYR, IFIH1, CD80, CLNK, BACH2, SLA, CASP7, CD44, IKZF4, SH2B3* and *TOB2*. Most vitiligo susceptibility loci encode immunoregulatory proteins or melanocyte components that may mediate immune targeting and the relationships among vitiligo, melanoma and eye, skin and hair coloration.

Table 4. Susceptibility loci for vitiligo.

Susceptibility loci	Chromosomal Region	Reference
SLEV1	17p13	Spritz *et al.*, 2004, Nath *et al.*, 2001
AIS1	1p31.3-p32.2	Alkhateeb *et al.*, 2002
AIS2	7p	Spritz *et al.*, 2004
FOXP1	3p13	Jin *et al.*, 2010b
MYH15	3q13.13	Jin *et al.*, 2010b
CCR6	6q27	Jin *et al.*, 2010b
ICA1	7p21.3	Jin *et al.*, 2010b
TBC1D2	9q22.33	Jin *et al.*, 2010b
IKZF4	12q13.2	Jin *et al.*, 2010b
SH2B3	12q24.12	Jin *et al.*, 2010b
RNASET2	6q27	Quan *et al.*, 2010
FGFR1OP	6q27	Quan *et al.*, 2010
CCR6	6q27	Quan *et al.*, 2010
IFIH1	2q24.2	Jin *et al.*, 2012
CD80	3q13.33	Jin *et al.*, 2012
CLNK	4p16.1	Jin *et al.*, 2012
BACH2	6q15	Jin *et al.*, 2012
SLA	8q24.22	

CASP7	10q25.3	Jin *et al.*, 2012
CD44	11p13	Jin *et al.*, 2012
TYR	11q21	Jin *et al.*, 2012
OCA2-HERC2	15q12-13.1	Jin *et al.*, 2012
MC1R	16q24.3	Jin *et al.*, 2012
TICAM1	19p13.3	Jin *et al.*, 2012
TOB2	22q13.2	Jin *et al.*, 2012

1.9 Candidate genes addressesd in the present study:

1.9.1 *ACE* (Angiotensin converting enzyme):

ACE, a key enzyme in the renin-angiotensin system. The gene encoding *ACE* (or dipeptidyl carboxy peptidase 1: DCP1) is of 24 kb consisting of 26 exons, and is located on chromosome 17q23. It catalyzes the conversion of angiotensin I to angiotensin II in kidney through removal of two C-terminal residues. Activation of ACE is as explained in Figure 10.The ACE plays an important role in the physiology of the vasculature, blood pressure and inflammation, and it has also been found to be associated with several autoimmune disorders (Vuk-Pavlovic *et al.,* 1988, Scholzen *et al.,* 2003).

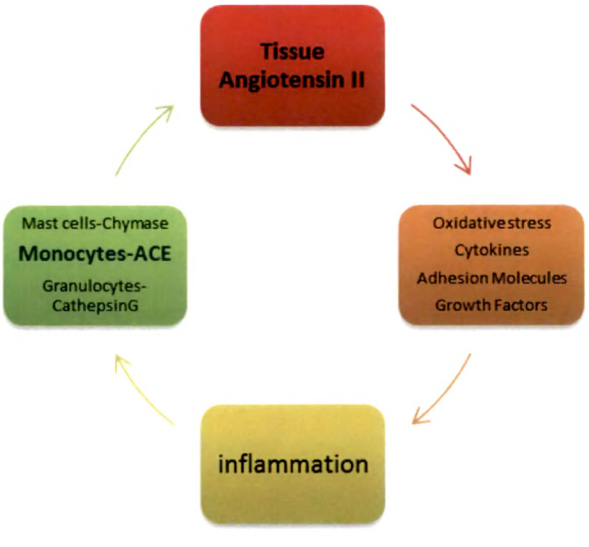

Figure 10. Activation of tissue ACE:

It creates a positive-feedback mechanism for tissue Ang II formation with subsequent induction of oxidative stress and inflammation. Activation of tissue ACE promotes release of cytokines and growth factors that increase vessel wall inflammation.

Inflammatory cells, in turn, release enzymes and other substances that generate Ang II. (Dzau *et al.*, 2001).

1.9.1.1 *ACE* I/D polymorphism:

An insertion/deletion (I/D) polymorphism of a 287-base pair repetitive sequence in intron 16 of the *ACE* gene (NCBI: AF118569; repeat region 14094–14381) has been associated with the development of vitiligo (Jin *et al.*, 2004; Deeba *et al.*, 2009; Tippisetty *et al.*, 2011). The insertion/deletion (I/D) polymorphism of the *ACE* gene accounts for a large proportion of the variability of serum ACE activity, D/D genotypes having the highest and I/I genotypes having the lowest ACE activity (Rigat *et al.*, 1990).

1.9.2 *CTLA4* (Cytotoxic T lymphocyte-associated antigen4):

CTLA-4, also known as CD152 is a type 1 transmembrane glycoprotein of the immunoglobin superfamily (Brunet *et al.*, 1987). The cytoplasmic portion of CTLA-4 lacks any intrinsic enzymatic activity (Baroja *et al.*, 2000). It contains a lysine-rich motif located proximal to the membrane (Baroja *et al.*, 2002). The *CTLA4* gene is located on chromosome 2 in humans (Dariavach *et al.*, 1988,). This gene consists of 4 exons (Dariavach *et al.*, 1988, Ling *et al.*, 1999) (Figure 11). The resulting transcript can undergo alternative splicing, which is different between human and mouse *CTLA4*. For human *CTLA4*, one can detect (a) full-length mRNA containing exons 1 to 4, (b) a transcript coding for soluble *CTLA4* (sCTLA-4) and (c) a transcript coding only for exons 1 and 4 (Brunet *et al.*, 1987, Dariavach *et al.*, 1988, Ling *et al.*, 1999, Ueda *et al.*, 2003, Vijayakrishnan *et al.*, 2004, Magistrelli *et al.*, 1999, Oaks *et al.*, 2000).

The *CTLA4* gene is mainly expressed in T cells upon activation (Perkins *et al.*, 1996), although it is constitutively expressed in the $CD4^+CD25^+$ regulatory T cell (Treg) subset (Lindsten *et al.*, 1993, Perkins *et al.*, 1996, Harper *et al.*, 1991, Takahashi *et al.*, 2000). Memory T cells have a relatively large pool of intracellular CTLA-4, which is rapidly expressed on the cell surface upon activation.The transcriptional regulation of *CTLA4* gene expression is only partially known. Sequence analysis of

the 5' upstream region of the *CTLA4* gene revealed several transcriptional regulatory elements, including sites for AP1, STAT, GATA1, NF-κB, Oct1, and an IL4 negative regulatory element (Ling *et al.,* 1999, Perkins *et al.,* 1996, Finn *et al.,* 1997). Also, *CTLA4* expression is upregulated by cAMP, suggesting a cAMP-dependent regulation of *CTLA4*gene transcription (Vendetti *et al.,* 2002).

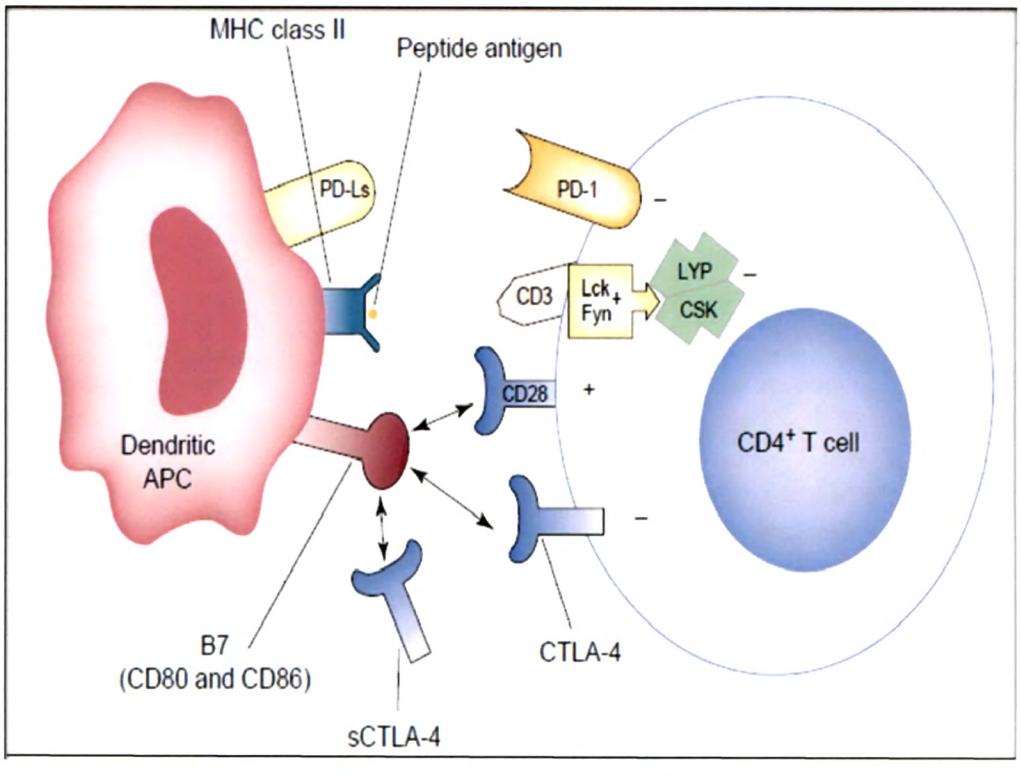

Figure 11: Interaction of CTLA4 and B7 molecules of Dendritic APC's.

(Kuby, *Immunology*, 6 *th Ed.*, 2007)

1.9.2.1 *CTLA4* polymorphisms:

Given the evolutionary conservation of *CTLA4* and its differential expression in T cell subsets, transcriptional or translational changes in its expression may have serious immunological consequences. This has justified the search for Polymorphisms and their link with immune-mediated diseases. To date, four main Polymorphisms of the *CTLA4* gene have been identified and studied in the context of autoimmune disorders. SNP's have been noted in human *CTLA4* at positions –1722, –1661, and –318, all in the regulatory/promoter region, and at position +49 in exon 1, which results in the substitution of an alanine for a threonine in the signal sequence of *CTLA4* (Nistico *et*

al., 1996, Deichmann *et al.,* 1996, Donner *et al.,* 1997, Wang *et al.,* 2002, Kristiansen *et al.,* 2000).

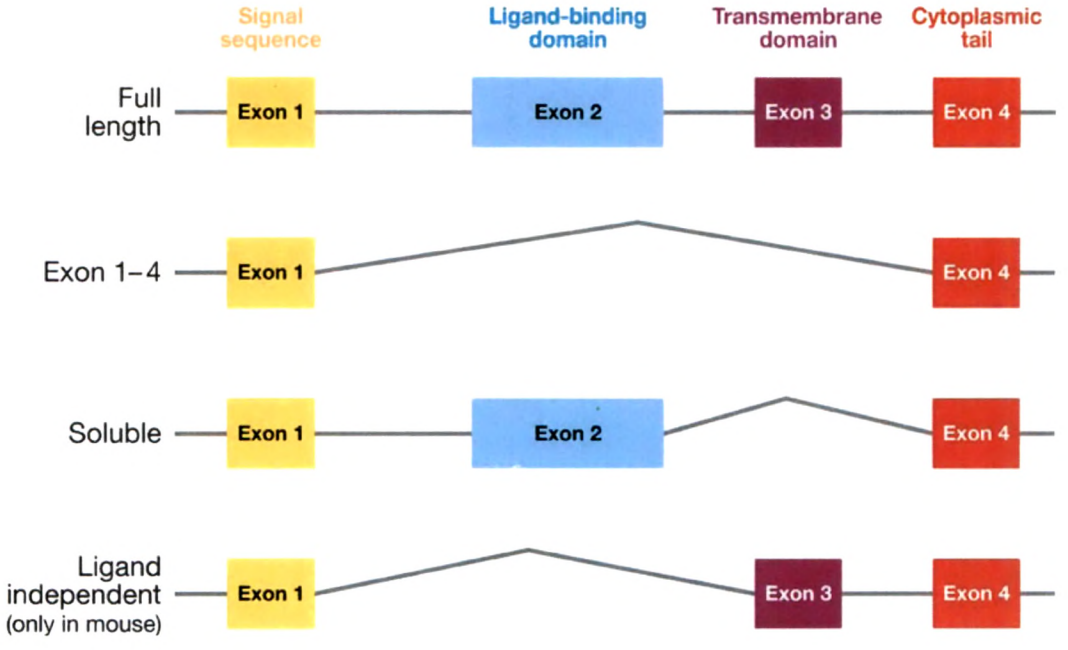

Figure 12: The *CTLA4* gene and its various splice variants.

The *CTLA4* gene consists of four exons that can give rise to four different splice variants. Each exon codes for a domain in the protein as indicated in the top of the figure. Humans express the full-length *CTLA4*, exon 1–4 *CTLA4*, and soluble *CTLA4*splice variants, whereas mice additionally express the ligand-independent *CTLA4*.

The A49G polymorphism is the only polymorphism that changes the primary amino acid sequence of *CTLA4*. In vitro studies of A49G *CTLA4* have shown that this mutant form of *CTLA4* is aberrantly processed in the endoplasmic reticulum, leading to reduced surface expression (Anjos *et al.,* 2002). In contrast, the C-318T polymorphism has been associated with higher promoter activity and subsequently increased *CTLA4* expression (Wang *et al.,* 2002). The other two Polymorphisms, T-1772C and A-1661G, are less characterized, and further investigation is required to define their effects on *CTLA4* expression and association with immunological pathologies. In addition, a dinucleotide repeat polymorphism in the 3UTR of *CTLA4*

has been identified (Huang *et al.,* 2000). Patients with longer AT repeats in the 3' UTR have T cells that have higher proliferative responses when stimulated with anti-CD3/anti- CD28 (Wang *et al.,* 2002). The correlation between all these polymorphisms of *CTLA4* and susceptibility to autoimmune disorders has been contentious, with some reports indicating strong correlation and others disputing such a correlation. More importantly, how these Polymorphisms affect CTLA4 function is unclear. They may affect CTLA4 processing and intracellular trafficking, or they may affect oligomerization and surface retention through indirect effects on CTLA4 posttranslation modifications.

Using the candidate gene approach, the role of CTLA4 has been investigated in various autoimmune diseases e.g. Graves disease, Hashimoto's thyroiditis (HT), Addison's disease (AD), Insulin-dependent diabetes mellitus (IDDM), Myasthenia gravis (MG), Multiple sclerosis (MS), Systemic lupus erythematosus (SLE), Rheumatoid arthritis (RA) including Vitiligo.

1.9.3 *PTPN22* (Protein tyrosine phosphatase non receptor 22):

The *PTPN22* gene, located on chromosome 1p13, encodes a lymphoid specific phosphatase (LYP), which is important in the negative control of T lymphocyte activation (Hill *et al.,* 2002). LYP is an intracellular PTP with a molecular weight of 110 kDa that contains an N- terminal catalytic domain and a non-catalytic C-terminus with 4 proline rich motifs. LYP physically bound through proline-rich motif to the SH3 domain of the Csk kinase, which is an important suppressor of T-cell receptor signaling kinases LCK and FYN that mediate T-cell activation (Cloutier *et al.,* 1999, Palacios *et al.,* 2004). The ability of Csk and LYP to inhibit T-cell receptor signaling requires their physical association (Cohen *et al.,* 1999).

Protein tyrosine phoshatases (PTPs) are critical regulators of T-cell signal transduction (Figure 13) (Bottini *et al.,* 2004, Smyth *et al.,* 2004). In conjunction with protein tyrosine kinases PTPs regulate the reversible phoshorylation of tyrosine residues and thereby play important roles in many different aspects of T-cell physiology (Alonso *et al.,* 2004). Abnormalities in tyrosine phosphorylation have been shown to be involved in the pathogenesis of numerous human diseases from

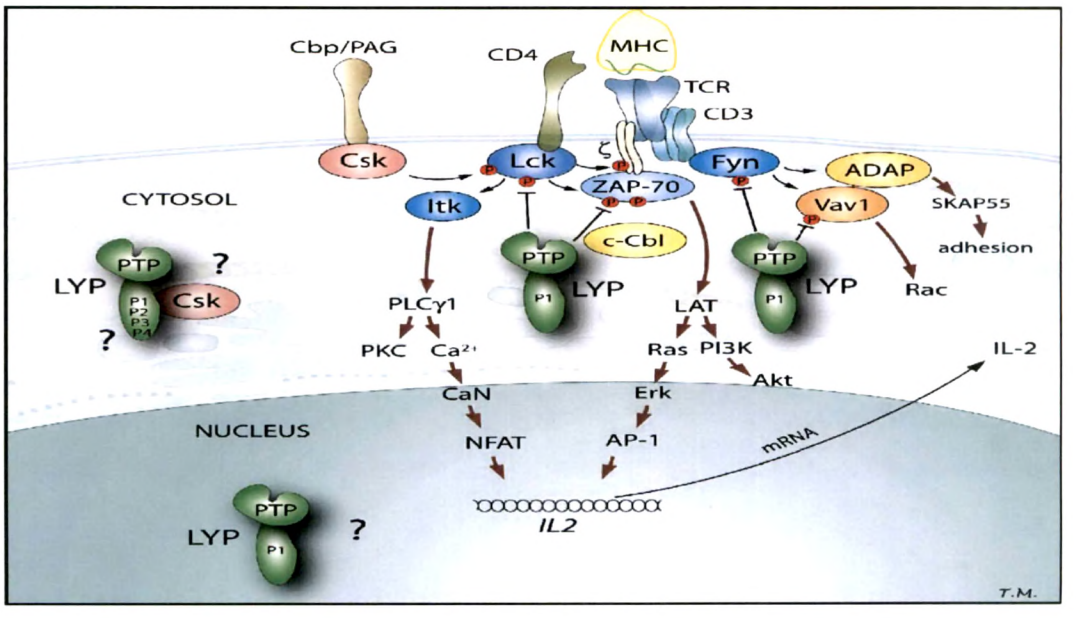

Figure 13: Role of *PTPN22* in T-cell receptor signaling (Bottini *et al.*, 2006)

1.9.3.1 *PTPN22* Polymorphism:

Recently, an association between the minor allele (T) of a missense single-nucleotide polymorphism [SNP; R620W (rs2476601, 1858C/T)] in the protein tyrosine phosphatase non-receptor type 22 gene (*PTPN22*) and susceptibility to generalized vitiligo has been described (Canton *et al.,* 2005). Besides its association with vitiligo the missense R620W polymorphism in the *PTPN22* gene at nucleotide 1858 (1858C/T) in codon 620 (620Arg→Trp) has been also associated with autoimmune diseases including type I diabetes mellitus, Graves' disease, systemic lupus erythematosus and rheumatoid arthritis (Bottini *et al.,* 2004, Smyth *et al.,* 2004, Onengut-Gumuscu *et al.,* 2004, Ladner *et al.,* 2004, Velaga *et al.,* 2004, Kyogoku *et al.,* 2004, Orozco *et al.,* 2005) suggesting a genetic predisposition towards generalized T-cell autoimmunity. Despite the association of *PTPN22* C1858T SNP with the multiple different autoimmune disorders, there are some autoimmune diseases such as psoriasis, IBD, MS, Addison's disease and Celiac diseases in which *PTPN22* C1858T SNP does not appear to play a role in susceptibility (Prescott NJ *et al.,* 2005, Martin MC *et al.,* 2005, Wagenleiter SE *et al.,* 2005, Matesanz F *et al.,* 2005, Ittah M *et al.,* 2005 and Gonzalez-Gay MA *et al.,* 2005).

The *PTPN22* 1858 C→T SNP changes the amino acid at position 620 from an Arginine(R) to a tryptophan (W) and disrupts the interaction between Lyp and Csk, avoiding the formation of the complex and therefore the suppression of T-cell activation. In vitro experiments have shown that the T-allele of *PTPN22* binds less efficiently to Csk than the C-allele does, suggesting that T-cells expressing the T-allele may be hyper responsive and consequently, individuals carrying this allele may be prone to autoimmunity (Cloutier JF *et al.,* 1999). The *PTPN22* 1858T variant has recently been described to result in a gain-of-function form of the enzyme leading to stronger suppression of the early T-cell activation process. T cells from individuals carrying the predisposing T-allele produced less interleukin-2 and encoded a phosphatase that had higher catalytic activity and thus represents a gain of function polymorphism (Vang *et al.,* 2005).

Experimental evidence suggests that the disease-associated LYP variant SNP lies within the first proline-rich domain of *PTPN22* and results in the substitution of tryptophan for arginine. Trp620 prevents the interaction of LYP with CSK . Consequently, the T-cell receptor-associated kinases might be able to induce T-cell activation in an uncontrolled manner and this may increase the overall reactivity of the immune system and may result in failure to delete autoreactive T cells during thymic selection or decreased activity of regulatory T cells. This in turn may predispose an individual to autoimmune disease.

1.9.4 *MBL* (Mannose binding lectin):

MBL is a serum protein produced in the liver, and is a key molecule in innate immunity. MBL, along with other molecules such as surfactant protein A (SP-A) and surfactant protein D (SP-D), is a member of the collectin family, the characteristic of which being possession of a carbohydrate recognition domain (CRD) and a collagenous domain [Holmskov *et al.,* 1994]. MBL is a trimer protein composed of 3 identical polypeptides with a molecular weight of around 32 kDa (228 amino acids). Each polypeptide consists of a CRD, a neck domain, a collagen domain and a cystein rich region. MBL binds to various organisms by its CRD, and excises an opsonin effect. The multimeric structure of MBL allows it to bind to various microorganisms including Gram positive and negative bacteria, mycobacterium, viruses and fungi.

The binding of MBL leads to agglutination of these microorganisms and will help their clearance by phagocytes. In addition, MBL activates the complement pathway (the lectin pathway) through mannose binding lectin associated serine proteases (MASPs). Therefore, MBL is important in host defense, especially in infancy, when the acquired immunity has not fully developed. Individuals lacking this protein may develop severe episodes of bacterial infections from early life [Koch *et al.,* 2001, Summerfield *et al.,* 1997]. MBL is also important when the immune system of an individual is compromised, such as when a patient is under immunosuppressive therapy, or receiving chemotherapies or bone marrow transplant [Mullighan *et al.,* 2004]. While MBL is an acute phase protein and its production is enhanced by inflammatory stimuli, Polymorphisms of the MBL gene is known to greatly influence serum MBL concentration. The MBL gene, located on the long arm of chromosome 10 at 10q11.2–q21, contains 4 exons [Sastry *et al.,* 1989].

1.9.4.1 *MBL2* Polymorphisms :

The functional *MBL2* gene is located on chromosome 10 (q11.2-q21) and comprises of four exons. Exon 1 encodes the signal peptide, a cysteine rich region and part of the glycine-rich collagenous region. Three functional single-nucleotide Polymorphisms (SNPs) in exon 1 of *MBL2* gene have been reported: codon 54 (GGC→GAC; designated B allele), codon 57 (GGA→GAA; designated C allele), and codon 52 (CGT→TGT; designated D allele) (Sumiya *et al.,* 1991; Lipscombe *et al.,* 1992; Hansen and Holmskov, 1998). These variant alleles in the collagen like domain cause structural changes in MBL and will lead to its low plasma levels and thus results in plasma concentrations varying by more than 1000-fold within the population (Garred *et al.,* 2003; Thiel, 2007). The promoter region of the *MBL2* gene contains a number of regulatory elements, which affect transcription of the gene and hence Polymorphisms in the promoter region, thus affecting the plasma MBL concentration directly. Besides these exon 1 variant alleles, SNPs at promoters -550 (G/C; allele L) and -221 (G/C; allele X) have also been associated with low plasma levels of MBL (Madsen *et al.,* 1995). An association between *MBL2* gene codon 54 (allele B) polymorphism and susceptibility to vitiligo has been described in Turkish population (Onay *et al.,* 2007).

1.9.5 *NALP1* :

The *NALP1* gene, mapped to chromosome 17p13, has been linked to vitiligo and associated autoimmunity (Jin Y *et al.,* 2007). The *NALP1* gene encodes NALP1, a leucine-rich repeat protein 1, a member of the nucleotide oligomerization domains-like receptors (NLRs) family. The NLRs are a group of cytoplasmic pattern recognition receptors, which nonspecifically recognize microbial products, such as lipopolysaccharide, thereby stimulating innate immunity.

The *NALP1* gene encodes NLRP1, a leucine-rich repeat protein 1, a member of the nucleotide oligomerization domains-like receptors (NLRs) family. The NLRs are a group of cytoplasmic pattern recognition receptors, which nonspecifically recognize microbial products, such as lipopolysaccharide, thereby stimulating innate immunity. NALP1 contains five domains, a protein-protein interaction N-terminal pyrin domain followed by centrally located NACHT (NAIP CIIA HET-E and TP1 family proteins) domain responsible for oligomerization and activation of the NLRs, a domain comprised of five tandem leucine-rich repeat (LRR) domains that is speculated to bind microbial ligands, a FIIND domain, and a finally a C-terminal caspase recruitment (CARD) domain (Martinon and Tschopp, 2005).

The primary function of human NALP1 is caspase activation. Upon activation by appropriate PAMPs, resulting in its oligomerization, human NALP1 binds adapter protein ASC via its N-terminal PYD. ASC, in turn, binds pro-caspase-1, forming a multiprotein complex termed an 'inflammasome' (Martinon *et al.,* 2002). The C-terminal CARD of NALP1 can also bind both pro-caspase-1 and caspase-5 (Tschopp *et al.,* 2003) (Figure 14). These members of the caspase family activate IL-1b and IL-18, as well as causing apoptosis, as highlighted previously. NALP1 may have other functions besides activation of caspase-1 in response to bacterial PAMP, MDP. In this regard, the CARD of NALP1 binds the CARD of Apaf-1, a caspase-9 activating protein that becomes activated by cytochrome c when released from mitochondria (Bruey *et al.,* 2007; Chu *et al.,* 2001) (Figure 15). Thus, it is possible that NALP1 contributes to apoptosis via caspase-9 activation in some contexts. Also, it is possible that Apaf1 and components of patched receptor complex are intrinsic activators of NALP1. Cytochrome c-mediated oligomerization of Apaf-1 conceivably could create

a platform for recruitment of NALP1, resulting in caspase- 1 activation via a mechanism whereby the Nterminal PYD domain of NLRP 1 binds ASC, which in turn binds pro-caspase-1, while the C-terminal CARD of NRLP1 binds the CARD of Apaf1. Activated patched receptors also could theoretically create an oligomerized protein complex at the plasma membrane for NRLP1 recruitment, thus promoting caspase activation.

1.9.5.1 *NALP1* Polymorphisms:

Given its location in the NALP1 protein, the missense mutation at L155H potentially could impact oligomerization of the protein, although other explanations are also possible. Polymorphisms in the promoter of the human *NALP1* gene presumably affect expression, but whether the result is increased versus decreased levels of NALP1 protein is unknown.

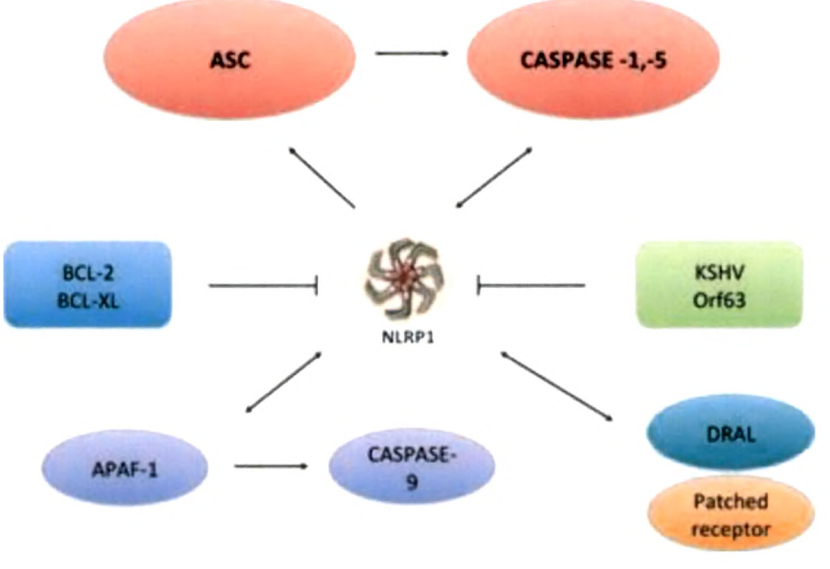

Figure 14. Protein interactions involving NALP1. Proteins known to interact with NALP1 are indicated, linking them to inflammation, apoptosis and other cellular functions.

Variations in the *NALP1* gene have been reported to confer risk for vitiligo and extended autoimmune disorders in Caucasian patients from the United Kingdom, the United States, and Romania (Jin Y *et al.,* 2007, Jin Y *et al.,* 2007). A recent report demonstrated that in a Norwegian population, Polymorphisms in *NALP1* are

associated with the organ-specific autoimmune conditions, Addison's disease, and type 1diabetes.

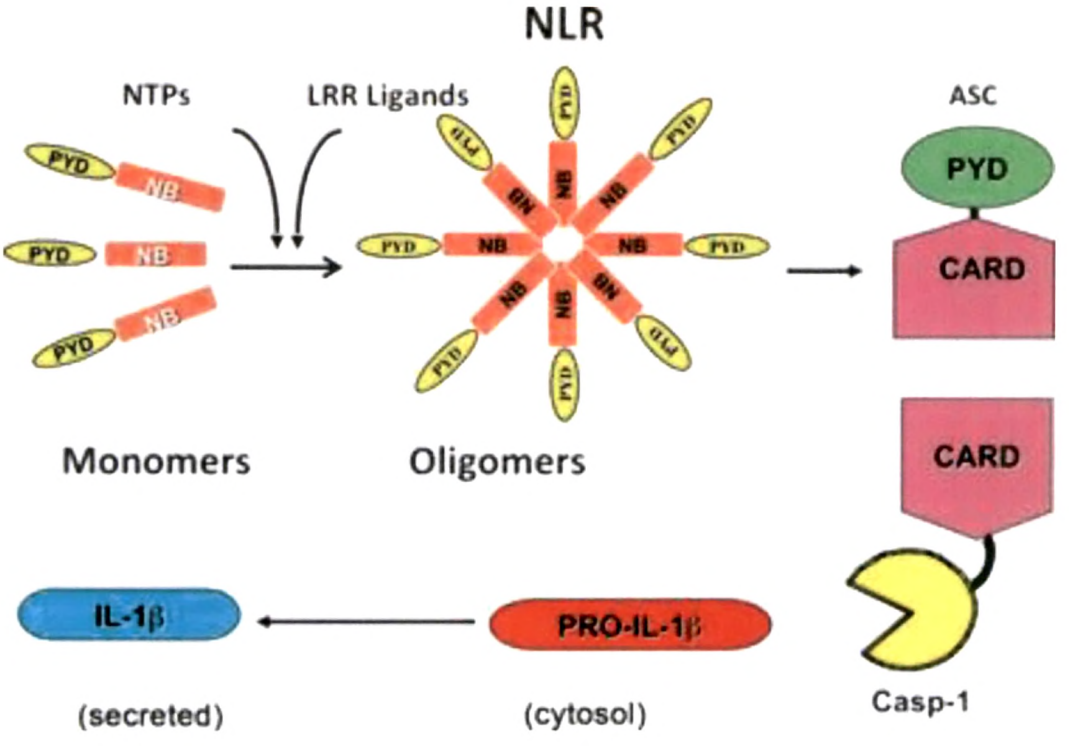

Figure 15. Inflammasome assembly. The steps involved in assembling the caspase-1-activating inflammasomes are depicted.

1.9.6 *MYG*1 (Melanocyte proliferating gene):

MYG1, C12orf10 in human is a ubiquitous nucleo-mitochondrial protein involved in early developmental processes (Philips *et al.,* 2009); however *MYG*1 expression in normal adult tissues is stable and seems to be changed mainly as a response to stress/illness conditions (Kingo *et al.,* 2006, Hawse *et al.,* 2003, Kõks *et al.,* 2004). MYG1 is predicted to possess metal-dependent protein hydrolase activity. MYG1 protein is highly conserved and present in all eukaryotes from yeast to humans (Nepomuceno-Silva *et al.,* 2004).

According to global expression analysis, *MYG1* tends to be up-regulated in undifferentiated and pluripotent cells. Kingo *et al.,* 2006 have suggested the involvement of *MYG*1 in vitiligo pathogenesis by showing elevated expression of *MYG1* mRNA in both uninvolved and involved skin of vitiligo patients. In addition,

MYG1 has been found to be consistently up-regulated also in skin biopsies from patients with atopic eczema (Sääf *et al.*, 2008).

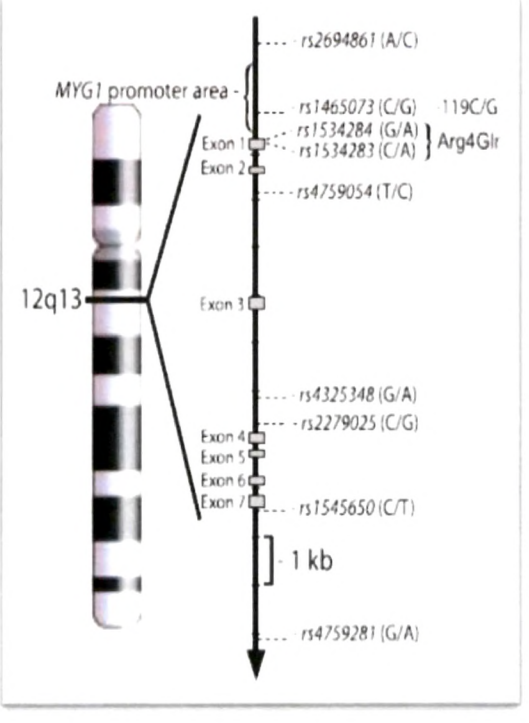

Figure 16: Relative positions of selective SNPs in *MYG* are represented by their ID numbers from NCBI's dbSNP database.

1.9.6.1 *MYG* Polymorphisms:

Philips *et al.*, (2010) have proposed that *MYG1* gene may be involved in vitiligo pathogenesis. This study demonstrated that both *MYG1* promoter polymorphism -119C/G and Arg4Gln polymorphism (Figure 17) in the mitochondrial signal of *MYG1* have a functional impact on the regulation of the *MYG1* gene and promoter polymorphism (-119C/G) is related with susceptibility for actively progressing vitiligo.

Philips *et al.*, (2010) showed that *MYG1* mRNA levels in skin samples of healthy controls correlate with *MYG1* promoter polymorphism -119C/G and subjects with homozygous -119G allele have significantly higher *MYG1* transcript levels than subjects with homozygous -119C allele. The study confirmed higher activity of -119G promoter by using *in vitro* luciferase reporter assay and showed alteration of p300

(E1A-associated 300 kDa protein) binding site is a potential reason why *MYG1* -119G promoter allele is more active. The -119G promoter was on average more than 2.5 fold more active regardless of the length of the promoter fragment (Philips *et al.*, 2010).

1.9.7 *IFNG* (Interferon γ):

Interferons are a small group of cytokines that include alpha, beta and gamma interferons (IFN-α,β,γ). Interferonγ, also known as type II interferon, is a single copy gene located on human chromosome 12q24 spans approximately 5.4 kb consisting of four exons and three introns (Gray *et al.*, 1982; Naylor *et al.*, 1983). It encodes a polypeptide of 166 amino acids with a 20 amino acid signal peptide and is an acid-labile protein which has antiviral, immune-regulatory and anti-tumour properties (Schroder *et al.*, 2004).

It alters transcription of up to 30 genes involved in a variety of physiological and cellular responses. IFNγ binding to IFNGR1 & IFNGR2 (interferon gamma receptors) activates the JAK-STAT pathway by phosphorylation of Jak and thereby activating STAT transcription factor (Horvath *et al.*, 2004). JAK-STAT signalling pathway is the best-characterised and commonly accepted IFN signalling pathway (Gough *et al.*, 2008). In addition, IFNγ activates antigen presenting cells (APCs) and promotes Th1 differentiation by up regulating the transcription factor and inhibits the development of Th2 cells (Oriss *et al.*, 1997). IFNγ is a pleiotropic cytokine that is a key regulator of development and functions of the immune system.

Apart from functions in host defense, IFNγ may also contribute to autoimmune pathology. Although IFNγ production was shown to be disease-limiting in autoimmune models such as murine experimental allergic encephalomyelitis (EAE) (Espejo *et al.*, 2001), it may contribute to autoimmune nephritis (Heremans *et al.*, 1978). In humans, IFNγ is implicated in pathology of diseases such as systemic lupus erythematosus (Lee *et al.*, 2001), multiple sclerosis (Panitch *et al.*, 1987), and insulin-dependent diabetes mellitus (Wang *et al.*, 1997).

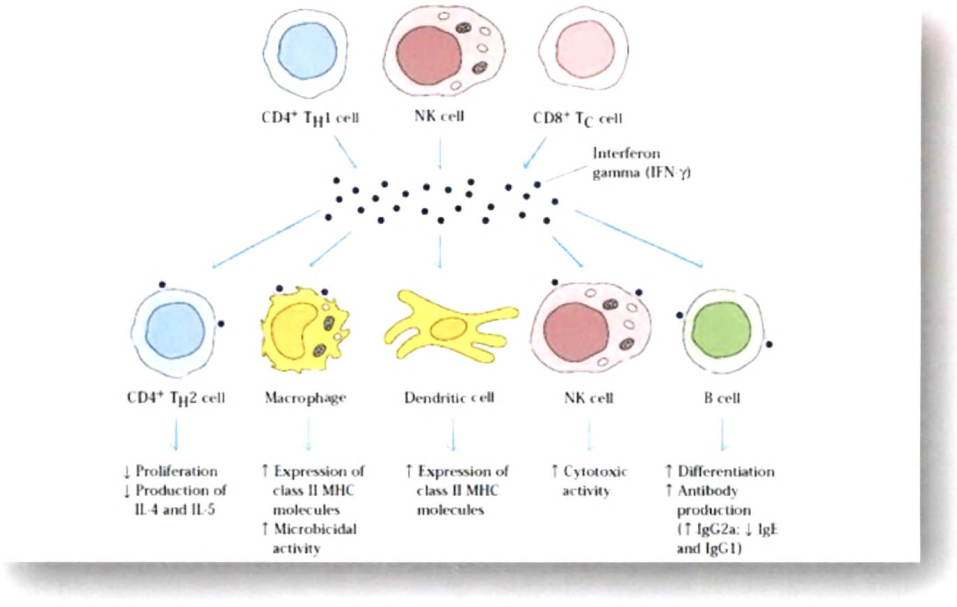

Figure 17: IFN-γ: Mode of Action (Kuby, *Immunology*, 6 th *Ed.*, 2007)

It has been suggested that melanocyte death is mediated by apoptosis in the context of autoimmunity and cytokines such as IFNγ and TNFα can initiate apoptosis (Huang *et al.,* 2002). Additionally, IFNγ and TNFα induce the expression of intercellular adhesion molecule-1 (ICAM-1) on the cell-surface of melanocytes (Yohn *et al.,* 1990). Increased expression of this adhesion molecule on the melanocytes enhances T cell/melanocyte attachment in the skin and may play a role in the destruction of melanocytes in vitiligo (Al Badri *et al.,* 1993).

The *IFNγ* coding region is invariant, with no reported polymorphisms (Hayden et al., 1997). There are two well-known single-nucleotide polymorphisms in the *IFNγ* gene. A CA repeat microsatellite sequence in the noncoding region of the first intron is polymorphic. Allele 2, with 12 CA repeats is associated with constitutively high IFNγ production in vitro. In addition, a single nucleotide A/T polymorphism at the 5' end of the CA repeat region in the first intron of the *IFNγ* gene (+874 A/T polymorphism; rs2430561) has been correlated with the presence or absence of the microsatellite allele 2 (Pravica *et al.,* 1999). Also, the presence of *IFNγ* (+874 Thi/Alo) polymorphism creates a putative NF-κB binding site in intron1 and shows preferential

binding to the T allele and correlates with high IFN-γ producer phenotype (Pravica *et al.*, 2000).

1.10 TREATMENT

Vitiligo is a difficult disease to treat. Although the treatment of vitiligo has improved during the last decade, it is still not satisfactory. Several treatment modalities are currently in use; however these methods usually induce incomplete pigmentation. Vitiligo treatment can be classified into two broad categories i.e. non-surgical therapies and surgical therapies (Nordlund *et al* 1993; Van Geel, 2001).

1.10.1 Non-surgical therapies:

1.10.1.1 Psoralen photochemotherapy:

Psoralenes are furocoumarin tricyclic hydrocarbon compounds. Psoralen photochemotherapy consists of photosensitizing psoralen with ultraviolet A in the 320-400 nm range (PUVA). PUVA and UVB therapies are widely used in the treatment of many skin disorders including vitiligo. The rationale of PUVA is to induce remissions of skin diseases by repeated controlled phototoxic reactions (Matsumura and Ananthaswamy, 2004). These reactions occur only when psoralenes are photoactivated by UVA. In systemic treatment, 8 methoxypsoralen or 4,5,8 trimethoxypsoralen is administrated before radiation exposure. The UV dosage is gradually increased until minimal erythema of vitiligo lesions occurs. How PUVA therapy stimulates the inactive melanocytes is unknown (Kovacs, 1998). The mechanism underlying the therapeutic effects of the combination of psoralen plus UVA is generally assumed that UVA induced DNA psoralen photoadducts impair the cell replication (Honig *et al* 1994). Inhibition of cell proliferation is observed at psoralen concentration and UVA doses which do not affect the cell viability (Luftl *et al.*, 1998); on the other hand higher doses cause irreversible DNA damage, resulting in both apoptosis and necrosis (Johnson *et al.*, 1996). It has been confirmed that the repigmentation is derived from the melanocyte reservoir in the hair follicles (Cui *et al.*, 1991). It has been demonstrated that PUVA irradiation of normal melanocytes *in vitro* inhibits the DNA and protein synthesis and affects EGF receptor and vitiligo associated melanocyte antigen expression. It is difficult to explain the PUVA induced

repigmentation of vitiligo on the basis of these different mechanisms. It has been proposed that PUVA could stimulate the production of melanocyte growth factor or may deplete antigens on vitiligo melanocytes, thus blocking the binding of specific autoantibodies, (Kao and Yu 1992). PUVA is immunosuppressive and this action of PUVA on T lymphocytes could be the reason for its therapeutic effect on vitiligo (Akyol *et al.,* 2002). It was proposed that PUVA inhibits gene transcription, which ultimately results in the shut down of cytokine release. Neuner *et al* showed the effect of PUVA on the release of the pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α from human peripheral blood mononuclear cells resulting in a significant reduction in these cytokines, thus causing the anti-inflammatory activity of PUVA (Neuner *et al* 1994).

1.10.1.2 Water bath PUVA:

The most recent model in phototherapy is water bath PUVA, in which the patient lies in a bath tub containing psoralen water for 15 min so that the drug gets absorbed on the skin and then goes for light therapy. This kind of therapy is especially beneficial in children for whom oral medicines are not safe (Aragane *et al.*, 2001). Another method of psoralen treatment, used rarely for pediatric patients with small, scattered vitiligo patches, involves the application of a very dilute solution of the drug directly to the affected skin area. This is then exposed to sunlight. Such topical treatment makes a person very liable to severe burn and blisters following too much sun exposure whereas water bath PUVA has the advantage of being done at home, and does not damage the entire skin surface.

1.10.1.3 Broadband UVB:

This phototherapy uses an emission spectrum of 290-320 nm (Koster and Wiskemann 1990).

1.10.1.4 Narrowband UVB:

In this phototherapy an emission spectrum of 310-315 nm is used (Westerhof and Korbotova 1997). Narrow band UVB therapy or TL-01 therapy is the latest in phototherapy for the treatment of vitiligo. In this therapy there is no need to take oral psoralen or apply psoralen. The therapy is very safe and can be safely administered

even to children. Narrow band UVB is much safer than full spectrum UVB. If exposure to natural sunlight is equal to 100% UV radiation , using a narrow band UV light is roughly 1% UV radiation . The advantage of UVB therapy over PUVA regimen is reflected by shorter duration of treatment (Van Geel *et al.,* 2001).

1.10.1.5 Topical immunomodulators:

Topical immunomodulatory agents such as tacrolimus and pimecrolimus offer several advantages in the treatment of vitiligo. These agents are well tolerated in children and adults and they can be used for long duration without evidence of atrophy or telangiectasias, the common complications associated with long term steroid use (Grimes, 2005). Tacrolimus is a topical immunomodulatory agent that affects T cell and mast cell functions by binding to cytoplasmic immunophilins and by inactivating calcineurin. Tacrolimus inhibits the synthesis and release of pro inflammatory cytokines and vaso active mediators from basophils and mast cells (Tharp, 2002). Pimecrolimus, which has a mechanism of action similar to tacrolimus, also can induce repigmentation in vitiliginous lesions (Mayoral *et al* 2003). As tacrolimus, pimecrolimus induces maximal repigmentation on sun-exposed areas.

1.10.1.6 Calcipotriol:

It is a synthetic analogue of vitamin D3. Vitamin D3 binds to vitamin D receptors in the skin, affecting melanocyte and keratinocyte growth and differentiation. It also inhibits T cell activation (Dusso and Brown 1998). Melanocytes express 1, 25-dihydroxyvitamin D3 receptors, which may stimulate melanogenesis.

1.10.1.7 Pseudocatalase:

The discovery of low epidermal catalase levels in involved and uninvolved skin of patients with vitiligo suggested a major stress arising from increased epidermal H_2O_2 generation (Schallreuter *et al* 1991). However, catalase mRNA levels in melanocytes and keratinocytes from patients is normal compared to healthy controls (Maresca *et al.*, 1997). One consequence of H_2O_2 accumulation is the oxidative degradation of the porphyrin active site of the catalase leading to its deactivation (Aronoff *et al.,* 1965).

Pseudocatalase is a bis (Mn) bicarbonate complex for the removal of H_2O_2 in the epidermis of vitiligo patients (Schallreuter *et al.,* 1995). Pseudocatalase functions as a pro-drug requiring UV light for the full activation of the complex (Schallreuter *et al.,* 1999a). Successful removal of the high levels of epidermal H_2O_2 in vitiligo was shown with a topical application of pseudocatalase in several studies (Schallreuter *et al.,* 1995). It has been demonstrated that *in vitro* and *in vivo* use of pseudocatalase leads to the recovery of $6BH_4$ whose recycling process is perturbed in vitiligo and thus leads to repigmentation (Schallreuter *et al.,* 2001).

1.10.1.8 Khellin and UVA:

Khellin is a furanochrome and combined with UVA, it is as effective as PUVA therapy in the treatment of vitiligo without having the phototoxicity associated with psoralens (Nordlund *et al.*, 1993).

1.10.1.9 Fake tanning products:

Cover creams or self tanning products are special drug cosmetics that can be used to match most skin patches when medical treatment is not successful. Patients with vitiligo are required to protect their depigmented skin against excessive sun exposure by wearing protective clothing. Tattooing is rarely recommended. It works best for the lip area, particularly in people with dark skin. However, it is difficult to perfectly match the skin, and tends to look worse over time. Cosmetics can be used to improve the appearance of the white areas not covered by clothing. Sunscreens give coolness to the affected areas and also prevent the normal skin around the patches from becoming darker. Bleaching or depigmentation of the normal skin and autologous transplantation of skin are an option for those who are severely affected (Samantha *et al.*, 2008).

1.10.2 Surgical therapies:

Several treatment modalities such as PUVA, -UVB and local corticosteroids are currently used in the treatment of vitiligo. However, these treatments usually induce incomplete repigmentation. Surgical methods intended to repigment vitiligo are an interesting therapeutic option if patients have stable disease (Ongenae *et al.,* 2001).

All surgical techniques have the same basic principle: to transplant autologous melanocytes from a pigmented donor skin to regions without melanocytes (Ongeneae *et al.,* 2001). Basically there are two types of surgical techniques, tissue grafts and cellular grafts. Tissue grafts are full thickness punch grafts and, split thickness grafts and suction blister grafts. With tissue grafts, only a limited surface area can be treated but with good results in the majority of cases.

1.10.2.1 Full thickness punch grafts:

In this method punch grafts from normally pigmented skin are implanted in the affected area. Repigmentation is based on the 'pigment spread phenomenon' by grafted piece of normal skin. The grafts are implanted into perforations previously made at the recipient site using a biopsy punch under local anesthesia (Ongenae *et al.*, 2001). The success rate of full thickness punch grafts is in between 68-82% (Malakar and Dhar 1999; Boersma *et al.,* 1995; Falabella *et al.,* 1988). Punch grafting is easy to perform and does not require special equipment or a laboratory set up. Difficult areas such as lips could be treated successfully; however it is not suitable for body folds (Malakar and Dhar 1999).

1.10.2.2 Split thickness grafts:

This technique has a high success rate of 78–91% (Olsson and Juhlin 1998; Kahn and Cohen 1998). After obtaining a split thickness skin graft using a dermatome it can be applied directly to the derma braded recipient area. Temporary small epithelial milia like cysts can be observed in the recipient area during the first months, especially on the face and neck. Scar or keloid formation at the donor site is reported in 12% of the patients treated with split thickness grafts. As donor tissue is limited more than one split skin grafting session can be necessary (Ozdemir *et al.*, 2002).

1.10.2.3 Suction blister grafts:

Grafts are carefully removed with sharp scissors and forceps after harvesting the graft. This epidermal sheet is then grafted onto the denuded recipient site. The success rate is 73–88%. Pigment spread after epidermal blister grafting can be enhanced by pre operative radiation therapy of the donor site using PUVA. Temporary hyper

pigmentation can be seen in the grafted sites in 2–65% (Ozdemir *et al.*, 2002). The eyelids, lips and bony prominences can be treated using this method.

1.10.2.4 Cultured epidermal grafts:

A shave biopsy of normally pigmented skin is the source of epidermal cell culture. The cultured sheet is released by treatment with dispase and attached to petroleum gauze as support. Subsequently the gauze to which the epithelium adheres will be applied onto the dermabraded recipient site and covered with occlusive dressing (Kumagai and Uchikoshi 1997). Success rate of this method is in between 33-54%.

1.10.2.5 Non-cultured keratinocytes and melanocytes:

Transplantation technique with a suspension of non cultured keratinocytes and melanocytes in the treatment of depigmented lesions is effective. Donor skin is obtained from the occipital area and immersed for 18 h in 0.25% trypsin solution. The following day the epidermis of the donor skin can be separated from the dermis *in vitro* using fine forceps. After several procedures a cellular suspension is obtained (Mysore and Salim, 2009). Liquid nitrogen is used to induce blisters in the recipient area. The cellular suspension from the donor site is injected into each blister at the recipient area after aspiration of the viscous blister fluid. The intact blister top is a natural dressing that holds the transplanted cells in place. It is important not to separate keratinocytes from melanocytes before grafting because factors furnished by keratinocytes sustain melanocyte growth (Ozdemir *et al.*, 2002). The success rate of this therapy is more than 70% (Gauthier and Surleve-Bazeille 1992).

1.10.2.6 Cultured melanocytes:

Lerner *et al.*, 1987 first described the use of cultured pure autologous human melanocytes. They cultured melanocytes of a shave biopsy from normally pigmented skin *in vitro* with the addition of several growth factors and chemical media. The success rates vary between 22-72%.

1.10.2.7 Depigmentation:

Depigmentation or the removal of remaining pigmentation is normally done in patients who have greater than 50% of their bodies affected and who have demonstrated recalcitrance to repigmentation. Depigmentation is permanent and irreversible. Monobenzylether of hydroquinone is used as a depigmenting agent (Nordlund *et al* 1993).

1.10.3 Herbal products

1.10.3.1 Anti-vitiligo® (True Herbals, Lahore, Pakistan):

Anti-vitiligo® (True Herbals, Lahore, Pakistan) is a traditional herbal formulation which was available internationally since November 2003. It is effective both in disease of recent onset as well as long standing established cases. Formulation contains the following ingredients.

1.10.3.2 Psoralea corylifolia:

It is a rich source of naturally occurring psoralen. It sensitizes human skin to the tanning effect of UV and sun light. *P. corylifolia* has been traditionally used both orally as well as in the form of topical preparations. Oxidative stress is widely believed to be one of the likely causative factors in the initiation of white skin patches of vitiligo. Hence, the protective, anti-oxidative and anti stress properties of *P. corylifolia* may contribute to the improvement in the hypo-pigmented white skin patches of vitiligo.

1.10.3.3 Black cumin:

Seeds of Nigella sativa have also been having an immunomodulatory as well as anti cancer effect, which is due to augmentation of T cell and natural killer cell mediated immune responses.

1.10.3.4 Barberry root:

Barberry root or the root of *Berberis vulgaris* contains numerous chemicals and bioactive compounds of medical significance. It contains for example the alkaloids like berbamine, berberine, and oxyacanthine. Other compounds include tannins,

chelidonic acid and resins. It is also quite rich in B-vitamin thiamine, lutein, vitamin C, beta-carotene, zeaxanthin, zinc, chromium, and cobalt. This herb has also been shown in scientific studies to possess antioxidant and cytoprotective properties.

1.10.3.5 Kalawalla® (American Life Style, New York, USA):

Kalawalla® (American Life Style, New York, USA) is a herbal product that works as a natural immunomodulator with proven immunomodulating effect. The product contains *Polypodium leucotomos* standardized extracts. *P. leucotomos* is a fern plant extract that has been used in Europe to treat vitiligo for over 10 years with encouraging results. The extract can help to regulate the immune system bringing it to its healthiest, strongest and balanced levels. Repigmentation results can be seen within the first month of taking the product. *P. leucotomos* standardized extract has been known to increase the lymphocyte levels. It is also known to regulate the CD4/CD8 ratios to their normal values.

1.10.3.6 Piperine:

The synthetic derivatives of piperine can stimulate pigmentation in the skin especially when combined with UVR treatment. The studies have compared the effects of piperine and its analogues tetrahydropiperine (THP), cyclohexyl analogue of piperine (CHP) and reduced CHP (rCHP) when applied to the skin of mice, either alone or followed by UV treatment. CHP did not show significant results while piperine, THP and rCHP did induce pigmentation in the skin. When used alone, the compounds stimulated pigmentation to an even, light brown color within six weeks. However, by accompanying the use of piperine or THP with UV, the skin became significantly darker, and within only seven weeks as compared to other treatments which take a year.

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CHAPTER II

EVALUATION OF AUTOIMMUNE HYPOTHESIS IN VITILIGO PATIENTS

2.1 INTRODUCTION

Vitiligo is an acquired depigmenting disorder characterized by the loss of melanocytes from epidermis. The mechanism of melanocyte loss from epidermis to cause vitiligo is not yet clearly understood (Taieb *et al.*, 2000). The three major hypotheses that are proposed to explain the pathogenesis of vitiligo are autoimmune, neurochemical, and oxidative stress hypotheses (Ortonne and Bose, 1993). Vitiligo is considered to be an autoimmune disease because of its association with several other autoimmune diseases such as diabetes, pernicious anemia, thyroid diseases, Addison's disease and alopecia areata (Kemp *et al.*, 2001). In addition, various circulating antimelanocyte and antikeratinocyte antibodies are found in vitiligo patients (Kemp *et al.*, 2001). A study performed on 2624 vitiligo probands from North America and UK confirmed significant increase in the frequencies of six autoimmune disorders in vitiligo probands and their first degree relatives: vitiligo itself, autoimmune thyroid disease (particularly hypothyroidism), pernicious anemia, Addison's disease, systemic lupus erythematosus and inflammatory bowel disease (Alkhateeb *et al.*, 2003). Association of these diseases with vitiligo indicates that vitiligo disorder shares common genetic etiologic link with other autoimmune diseases (Passeron and Ortonne, 2005). Also the immune suppressive effect of a number of repigmenting therapies (steroids) indirectly supports the autoimmune mediated process of depigmentation (Ongenae *et al.*, 2003).

The aim of the present study was to evaluate autoimmune hypothesis in vitiligo patients by estimating antimelanocyte antibody levels in patients and unaffected controls. The antimelanocyte antibody levels were also analyzed based on the onset of disease and disease progression.

2.2 MATERIALS AND METHODS

2.2.1 Study subjects:

The study group included 300 vitiligo patients [223 generalized (including acrofacial vitiligo and vitiligo universalis) and 77 localized vitiligo cases] comprised of 138 males and 162 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based

on the presence of depigmented patches on the skin and patients had no other associated autoimmune disease. A total of 400 ethnically sex-matched unaffected individuals (188 males and 212 females) were included in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. Importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 300)	(n = 400)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	138 (46.0%)	188 (47.00%)
Female	162 (54.0%)	212 (53.00%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Type of vitiligo		
Generalized	223 (74.33%)	NA
Localized	77 (25.67%)	NA
Active vitiligo	215 (71.67%)	NA
Stable vitiligo	85 (28.33%)	NA
Family history	41 (13.66%)	NA

2.2.2 Estimation of antimelanocyte antibody levels:

In the present study, plasma from vitiligo patients was examined for the reactivity with the human melanoma cell line (SK Mel 28) to find the levels of antimelanocyte antibodies in vitiligo patients compared to controls. We have analyzed plasma samples of 300 vitiligo patients and 400 controls for the presence of antimelanocyte antibodies by ELISA. 5 ml venous blood was collected from the patients and healthy

subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA) and plasma was extracted. Human melanoma cell line SK Mel 28 was obtained form NCCS, Pune and grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 50 mg/L gentamycin at 37°C under 5% CO_2 in a humidified atmosphere (Hann and Kim, 1995).

2.2.3 Enzyme linked immunosorbent assay (ELISA):

Cells were harvested by scraping and lysed by adding lysis buffer (HEPES 20 mM, EGTA 1 mM, PMSF 1 mM, $MgCl_2$ 1.5 mM, NaCl 150 mM, $CuSO_4$ 1mM, 1%,Trition –X 100, 1%, Glycerol). Protein estimation in the lysate was done by Lowry's method (Lowry *et al.,* 1951). 50 µl of the cell lysate containing 1 µg protein was loaded in each well of the microtiter plate and kept at 4° C overnight. Excess antigen was discarded, washed 3 times with PBS, blocked with 1% BSA in PBS and incubated for 1 hour. Excess blocking reagent was discarded and washed 3 times with PBS. 50 µl of plasma (1:10,000 diluted in PBS) was added to the microtiter plates and incubated for 2 hours at room temperature. Excess plasma was discarded and washed 3 times with PBS containing 0.2% Tween-20. Then 50 µl of 1: 2000 diluted secondary antibody was added (Rabbit anti human IgG HRP conjugate, Bangalore Genei, India) and incubated for 1 hour. Excess antibody was discarded and washed 3 times with PBS containing 0.2% Tween 20. 50 µl substrate (TMB-H_2O_2) was added and incubated for 5 minutes for the color development. The reaction was stopped by adding 200 µl of 1N H_2SO_4 and OD was read at 405 nm.

2.2.4 Statistical analysis:

Antimelanocyte antibody levels were plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.05 were considered as statistically significant.

2.3 RESULTS

2.3.1 Antimelanocyte antibody levels in vitiligo patients and controls:

Our results suggest significant increase in antimelanocyte antibody levels in vitiligo patients as compared to controls ($p<0.0001$; Figure1 A). We found that 75% of

vitiligo patients had antimelanocyte antibodies in their circulation suggesting that autoimmunity may play an important role in vitiligo pathogenesis.

2.3.2 Antimelanocyte antibody levels in different types of vitiligo patients:

Antimelanocyte antibody levels were also compared between generalized and localized vitiligo patients. Generalized vitiligo patients showed significant increase in antimelanocyte antibody levels as compared to localized vitiligo patients (p=0.001; Figure 1B). Moreover, when antimelanocyte antibody levels were compared between active and stable cases of vitiligo, active vitiligo patients exhibited higher levels of antimelanocyte antibody as compared to stable vitiligo patients (p<0.0001; Figure 1C).

2.3.3 Gender biasness in antimelanocyte antibody levels in vitiligo patients:

Furthermore, when antimelanocyte antibody levels were compared based on the gender of patients, female patients showed significantly increased levels of antimelanocyte antibodies as compared to male patients (p=0.003; Figure 1D).

2.3.4 Antimelanocyte antibody levels in different age of onset groups of vitiligo:

When effect of antimelanocyte antibody levels on vitiligo onset was analyzed between different age of onset groups, we found that patient group with an early age (1-20 yrs) of onset had significantly higher levels of antimelanocyte antibodies in their circulation as compared to the age groups 21-40 yrs (p=0.0004), 41-60 (p<0.0001) and 61-80 yrs (p<0.0001) (Figure 1E).

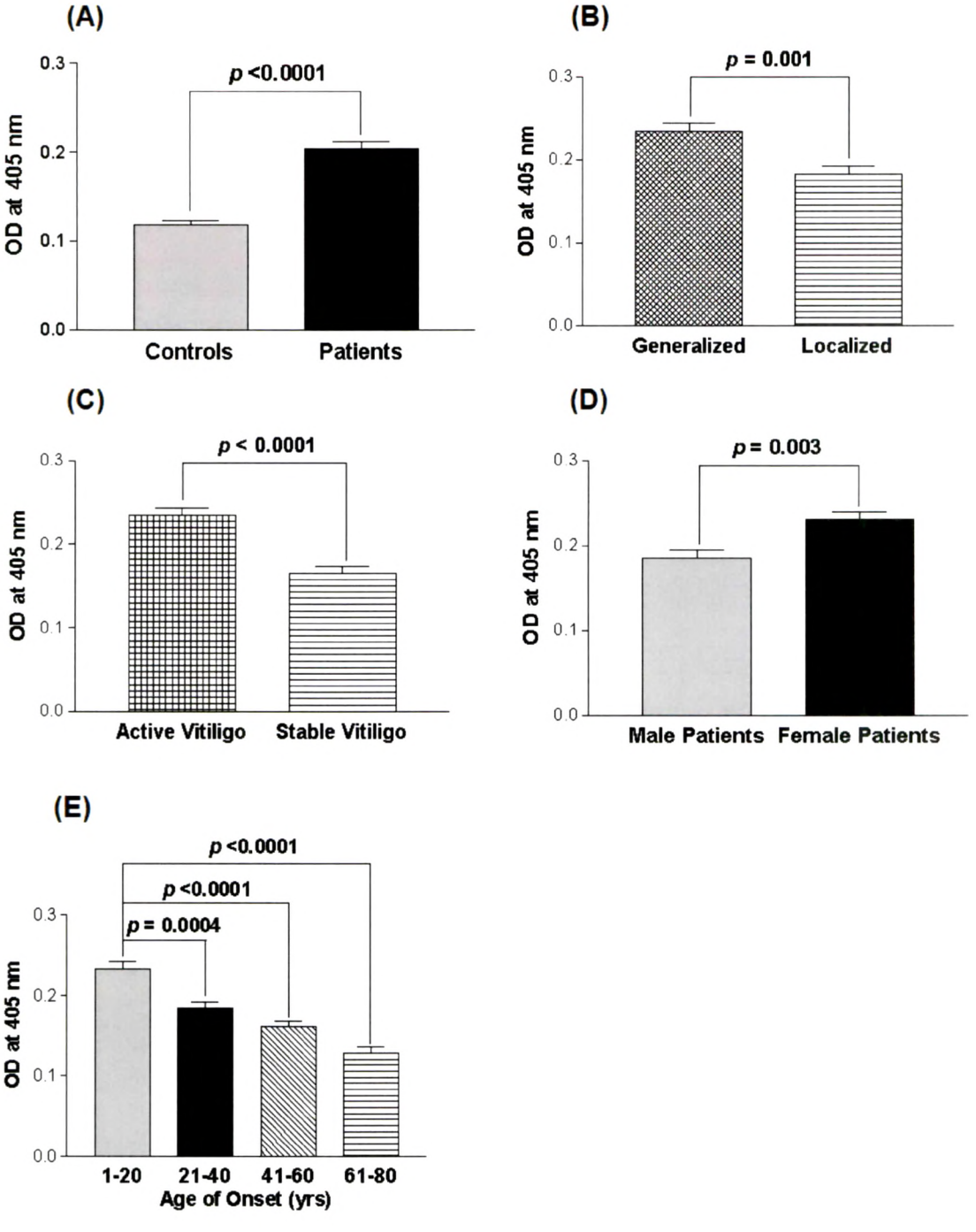

Figure 1. Antimelanocyte antibody levels in vitiligo patients and controls:

(A) Antimelanocyte antibody levels in 300 vitiligo patients and 400 controls. Patients showed significantly higher levels of antimelanocyte antibody as compared to controls (Mean ± SEM: 0.2029 ± 0.0080vs 0.1186 ± 0.0042; $p<0.0001$).

(B) Antimelanocyte antibody levels in 223 generalized and 77 localized vitiligo patients. Patients with generalized vitiligo showed significantly higher levels of

antimelanocyte antibody as compared to localized vitiligo (Mean ± SEM: 0.2338 ± 0.0105 vs 0.1830 ± 0.0090; p=0.001).

(C) Antimelanocyte antibody levels in 215 active and 85 stable vitiligo patients. Patients with active vitiligo showed significantly higher levels of antimelanocyte antibody as compared to stable vitiligo (Mean ± SEM: 0.2350 ± 0.0086 vs 0.1653 ± 0.0079; p<0.0001).

(D) Antimelanocyte antibody levels in 138 male and 162 female vitiligo patients. Female patients showed significantly higher levels of antimelanocyte antibody as compared to male patients (Mean ± SEM: 0.2306 ± 0.0096 vs 0.1853 ± 0.0099; p=0.003).

(E) Antimelanocyte antibody levels with respect to different age groups in 300 vitiligo patients. Vitiligo patients with the age of onset 1-20 yrs showed significantly increased antimelanocyte antibody levels as compared to the age groups 21-40 yrs (Mean [redacted] ± SEM: 0.2323 ± 0.0089 vs 0.1830 ± 0.0091; p=0.0004), 41-60 (Mean [redacted] ± SEM: 0.2323 ± 0.0089 vs 0.1606 ± 0.0076; p<0.0001) and 61-80 yrs (Mean [redacted] ± SEM: 0.2323 ± 0.0089 vs 0.1280 ± 0.0084; p<0.0001).

2.4 DISCUSSION

Vitiligo is strongly associated with other autoimmune conditions, such as type 1 diabetes, pernicious anemia, Addison's disease, systemic lupus erythematosus, alopecia areata, rheumatoid arthritis, and polyglandular autoimmune disease. Histological evidence further supports an autoimmune etiology. Michelsen (2010) has proposed antibody-based dominant mechanism in generalized vitiligo as a contributory factor for precipitation of autoimmune vitiligo. Antibodies (mainly IgG) against melanocyte antigens are detected in the sera of vitiligo patients and a correlation exists between the level of melanocyte antibodies and disease intensity in vitiligo patients (Harning *et al.*, 1991). Further, a correlation between the presence of antibodies and the extent of disease was established, as antibodies are present in more than 90% of patients with greater depigmentation and in 50% with minimal lesions (Abu Tahir *et al.*, 2010).

Interestingly, deposits of IgG have been observed in the basal membrane zone of lesional skin in relation to increased intensity of disease, which correlates well with

the observation of IgG binding to cultured melanocytes. Further studies have shown even the presence of anti-melanocyte IgA antibodies associated with disease manifestation (Ongenae et al., 2003, Kemp et al., 2007). These findings clearly suggest a vitiligo associated link with alterations in immunoglobulins.

The results of present study indicate that 75% of Gujarat vitiligo patients exhibited antimelanocyte antibodies in their circulation. Vitiligo patients showed significant increase in the levels of antimelanocyte antibodies in their circulation compared to controls (Figure 1A). Naughton *et al.*, (1983a) reported that 82% of vitiligo patients showed antimelanocyte antibodies in their circulation. In another study, 12/12 vitiligo patients and 0/12 controls were reported to have antimelanocyte antibodies in their circulation (Naughton *et al.*, 1983b). However, Grimes *et al.*, (1983) reported that 19% of Black vitiligo patients showed antimelanocyte antibodies in their circulation. Farrokhi *et al.*, (2005) also showed the presence of antimelanocyte antibodies in 30.9% of Iranian vitiligo patients. These reports suggest the involvement of antimelanocyte antibodies in pathogenesis of vitiligo.

Further we have analyzed antimelanocyte antibody levels in different groups of vitiligo to find whether antimelanocyte antibodies play a significant role in a particular type of vitiligo. Interestingly, we found that generalized vitiligo patients have significantly increased levels of antimelanocyte antibodies compared to localized vitiligo. This finding also supports the fact that generalized vitiligo has an autoimmune origin and is now considered as 'Autoimmune Vitiligo' (Nordlund *et al.*, 2006). Furthermore, our analysis based on progression of vitiligo suggested increased antimelanocyte antibodies levels in active cases as compared to stable cases suggesting the crucial role of antimelanocyte antibodies in disease progression. Interstingly, patients with an early onset of vitiligo exhibited significantly higher levels of antimelanocyte antibodies as compared to late onset patients. This suggests that antimelanocyte antibodies play an important role in early onset of the disease.
In addition, anaysis based on gender suggested higher antimelanocyte antibodies levels in female patients as compared to male patients indicating that females have increased susceptibility towards vitiligo as compared to males, implicating gender biasness in the development of autoimmunity (Whitacre, 2001; Panchanathan and Choubey, 2012; Afshan *et al.*, 2012).

The exact role of antimelanocyte antibodies in the pathogenesis of vitiligo remains unresolved. Autoantibodies against pigment cells might result from a genetic predisposition to immune dysregulation at the T cell level (Kemp *et al.,* 2001). Alternatively cross-reacting antigens expressed either on other target cells or infecting microorganisms could elicit their production. Autoantibodies could also result from an immune response to melanocyte antigens released following damage to pigment cells by other mechanisms, and these antibodies might then exacerbate the condition. The selective destruction of melanocytes might result from antibody reactivity directed to the antigens preferentially expressed on pigment cells (Kemp *et al.*, 1997) or from an antibody response against antigens expressed on a variety of cell types (Cui *et al.*, 1992) that might selectively destroy melanocytes because they are intrinsically more sensitive to immune mediated injury than other cells (Norris *et al.*, 1988).

Tyrosinase is the principal antigen recognized by these antibodies (Fishman *et al.*, 1993; Song *et al.*, 1994). The other melanocyte antigens recognized by autoantibodies are gp100/Pmel 17 (a melanosomal matrix glycoprotein), and tyrosinase related proteins 1 and 2 (TRP 1 and TRP 2) (Kemp *et al.*, 1998b). These cell differentiation antigens are localized primarily to melanosomes (Nordlund *et al.*, 1999). The transcription factors SOX9 and SOX10 are also identified as melanocyte autoantigens (Hedstrand *et al.*, 2001). Also autoantibodies against HLA Class I molecules are reported in vitiligo (Ongenae *et al.*, 2003). *In vitro* studies showed that autoantibodies are able to destroy melanocytes by complement mediated damage and antibody dependent cellular cytotoxicity (Gilhar *et al.,* 1995; Norris *et al.,* 1988; Cui *et al.*, 1993).

Moreover, autoantibodies could result from a failure to kill an autoreactive cell or by inducing autoimmunity against apoptotically modified cellular constituents. Therefore, the process of apoptosis may provide a source of cellular antigens to drive the autoantibody response and provide antigens. Reports have suggested that apoptosis is abnormal in autoimmune diseases and may play a role in the induction of autoimmunity. It is worth noting that efficient clearance of apoptotic cells is crucial for the avoidance of autoimmune responses to intracellular antigens (Casiano and Pacheco, 2006).

In conclusion, our results suggest significant increase in antimelanocyte antibody levels in vitiligo patients as compared to controls. However, it is unclear whether antimelanocyte antibodies are the cause or effect of the disease.

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CHAPTER III A

GENETIC ASSOCIATION OF PROTEIN TYROSINE PHOSPHATASE NON-RECEPTOR TYPE 22 *(PTPN22)* GENE POLYMORPHISM WITH GENERALIZED VITILIGO SUSCEPTIBILITY

3A. INTRODUCTION

Vitiligo is a common dermatological disorder of the epidermis and hair follicles, manifested clinically as expanding hypopigmented lesions of the skin. The clinical hallmark of vitiligo is loss of melanin pigment due to loss of melanocytes in the lesional skin. It affects 0.5-1% of the world population (Taieb *et al.,* 2007). The incidence of vitiligo is found to be 0.5-2.5% in India (Handa and Kaur, 1999) wherein Gujarat and Rajasthan states have the highest prevalence ~8.8% (Valia and Dutta, 1996). The Gujarat population may be susceptible to this disease due to multiple reasons. The three prevailing hypotheses for the pathogenesis of vitiligo are the neurochemical, autoimmune and oxidative stress hypotheses. However, none of them explains the entire spectrum of this disorder.

Although the etiology of vitiligo remains obscure, autoimmunity has been suggested to play a major role in the pathogenesis of generalized vitiligo (Nordlund *et al.,* 2006; Kemp *et al.,* 2001). Support for this theory arises from the frequent association of vitiligo with other autoimmune disorders (Alkhateeb *et al.,* 2003; Laberge *et al.,* 2005) and the demonstration of autoantibodies to melanocyte proteins in the serum of patients with the disease (Kemp *et al.,* 1997; Shajil *et al.,* 2006b). Moreover, autoreactive cytotoxic T lymphocytes (CTLs), which specifically recognize melanocyte differentiation antigens, have been detected in both the peripheral blood and perilesional skin of individuals with vitiligo (Palermo *et al.,* 2001).

The *PTPN22* gene, located on chromosome 1p13, encodes lymphoid protein tyrosine phosphatase (LYP), which is important in the negative control of T lymphocyte activation (Hill *et al.,* 2002). Lymphoid protein tyrosine phosphatase is expressed in T lymphocytes and associates with C-terminal Src kinase (CSK) to form a complex that suppresses the T-cell receptor signaling kinases LCK and FYN (Palacios *et al.,* 2004). The *PTPN22* 1858T variant has been described to result in a gain-of-function form of the enzyme leading to stronger suppression of the early T cell activation process. T cells from individuals carrying the predisposing T allele produced less interleukin-2 and encoded a phosphatase that had higher catalytic activity and thus represents a gain of function polymorphism (Vang *et al.,* 2005).

The disease-associated LYP variant SNP lies within the first proline-rich domain of LYP and results in the substitution of tryptophan for arginine. Trp620 prevents the interaction of LYP with CSK (Bottini *et al.,* 2004). Consequently, the T-cell receptor-associated kinases might be able to induce T-cell activation in an uncontrolled manner and this may increase the overall reactivity of the immune system and may result in failure to delete autoreactive T cells during thymic selection or decreased activity of regulatory T cells, which in turn may predispose an individual to autoimmune disease.

Previously, an association between the minor allele (T) of a missense single-nucleotide polymorphism (SNP) 1858C/T in the protein tyrosine phosphatase non-receptor type 22 gene (*PTPN22*) and susceptibility to generalized vitiligo was described (Canton *et al.,* 2005). Besides its association with vitiligo this missense 1858C/T polymorphism is also reported to be associated with autoimmune diseases including type I diabetes mellitus, Graves' disease, systemic lupus erythematosus and rheumatoid arthritis (Bottini *et al.,* 2004; Orozco *et al.,* 2005) suggesting a genetic predisposition towards generalized T cell autoimmunity. Since generalized (nonsegmental) vitiligo is considered to have an autoimmune etiology, the present study investigated *PTPN22* 1858C/T (R620W; rs2476601) polymorphism in generalized vitiligo patients and ethnically age and sex matched controls.

3A.1 MATERIALS AND METHODS

3A.1.1 Study subjects:

The study group included 126 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 54 males and 72 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 140 ethnically age and sex-matched unaffected individuals (61 males and 79 females) were included as controls in the study. None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of generalized vitiligo patients and controls.

	Vitiligo Patients	**Controls**
	(n = 126)	(n = 140)
Average age (mean age ± SD)	30.34 ± 11.13 yrs	28.67 ± 12.37 yrs
Sex: Male	54 (42.86%)	61 (43.57%)
Female	72 (57.14%)	79 (56.43%)
Age of onset (mean age ± SD)	20.78 ± 12.71 yrs	NA
Duration of disease (mean ± SD)	7.32 ± 6.14 yrs	NA
Family history	17 (13.49%)	NA

3A.1.2 Genomic DNA preparation:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analysis.

3A.1.3 Genotyping of *PTPN22* 1858C/T (R620W; rs2476601) polymorphism:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype 1858C/T polymorphism of *PTPN*22 gene (Figure 1). The primers used for genotyping were: 5'-GCCTCAATGAACTCCTCAA-3' (forward) and 5'-CCTCCTGGGTTTGTACCTTA-3' (reverse). The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 94°C for 10 minutes followed by 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on 2.0% agarose gel stained with ethidium bromide.

Restriction enzyme: *Xcm*I (New England Biolabs, Beverly, MA) was used for digesting amplicons of 1858 C/T polymorphism of *PTPN*22 gene. 5 µL of the amplified products were digested with 5 U of the corresponding restriction enzyme in a total reaction volume of 25 µL, according to the manufacturer's instructions. The digestion products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.0% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

3A.1.4 Statistical analysis:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *PTPN22* 1858C/T SNP in patients and controls by comparing the observed and expected frequencies of genotypes using chi-squared analysis. Distribution of the genotypes and allele frequencies of 1858C/T polymorphism for patients and control subjects

were compared using the chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). p-values less than 0.05 were considered as statistically significant.

3A.2 RESULTS

3A.2.1 Analysis of association between *PTPN22* 1858 C/T polymorphism and susceptibility to vitiligo:

PCR-RFLP for *PTPN22* 1858 C/T polymorphism yielded a 400 bp undigested product corresponding to C allele and 238 bp and 162 bp digested products corresponding to T allele. The three genotypes identified by 2.0% agarose gel electrophoresis were: CC homozygous, CT heterozygous and TT homozygous for 1858 C/T polymorphism of *PTPN22* gene (Figure 1).

The genotype and allele frequencies of the 1858 C/T polymorphism in 126 vitiligo patients and 140 controls are summarized in Table 2. The *PTPN22* 1858 C/T polymorphism was not found to be in significant association with vitiligo patients (p=0.198) when genotypes were compared using chi-squared test-3x2 contingency table (Table 2). Also there was no significant difference in the allele frequencies for this polymorphism between patients and controls (p=0.560) (Table 2). The observed allele frequencies of C and T were 0.98 and 0.02 respectively in controls; 0.99 and 0.01 respectively in vitiligo patients (Table 2). The distribution of *PTPN22* 1858 C/T genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as patient groups (p=0.932 and p=0.994 respectively) (Table 2).

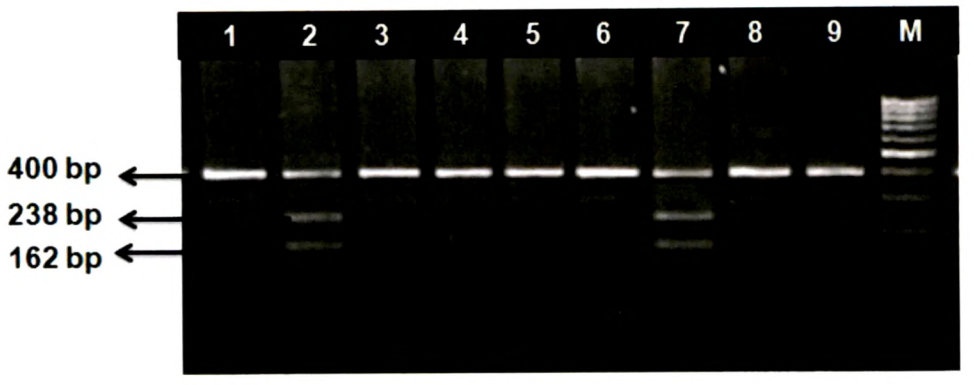

Figure 1. PCR-RFLP analysis of *PTPN22* 1858C/T SNP: 2.0 % agarose gel electrophoresis after overnight digestion of the PCR product with *Xcm*I. Lanes:1, 3, 4, 5, 6, 8 & 9 show subjects with homozygous CC genotypes; lanes: 2 & 7 show subjects with heterozygous CT genotypes. Lane: M shows a 100-bp ladder. No subject with homozygous TT genotypes was observed.

Table 2. Distribution of genotypes and alleles for the 1858C/T SNP of *PTPN22* in vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Associ-ation	*p* for HWE
	Genotype	(n = 126)	(n = 140)		
	CC	124 (0.98)	134 (0.96)		
PTPN22	CT	02 (0.02)	06 (0.04)	0.198	0.994[a]
1858 C/T	TT	00 (0.00)	00 (0.00)		(P)
(rs2476601)	Allele				0.932[b]
	C	250 (0.99)	274 (0.98)	0.560	(C)
	T	02 (0.01)	06 (0.02)		

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table, Values are significant at $p \leq 0.05$.

3A.3 DISCUSSION

The importance of genetic factors for vitiligo susceptibility is evident by reports of significant familial association (Bhatia *et al.,* 1992; Nordlund, 1997). About 20% of vitiligo patients have at least one first-degree relative affected (Bhatia et al, 1992; Nath *et al.,* 1994; Alkhateeb *et al.,* 2003) indicating that genetic predisposition may be involved in the vitiligo susceptibility. Our previous study also suggests that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil *et al.,* 2006b). The present study also includes 13.49% Gujarat vitiligo patients with positive family history (Table 1). The inheritance pattern of vitiligo does not follow the simple Mendelian pattern and its mode of heredity suggests that it is a polygenic disease. Vitiligo seems to be a complex hereditary disease governed by a set of recessive alleles situated at several unlinked autosomal loci, which may be involved in the antioxidant defense mechanism, melanin synthesis, autoimmunity etc. that could collectively confer the vitiligo phenotype (Nath *et al.,* 1994). Several candidate genes are reported for vitiligo (Fain *et al.,* 2003; Birlea *et al.,* 2011); however, the role of genetic factors in vitiligo pathogenesis is still not well understood.

PTPN22 gene has been found to play an important role in negative T cell regulation. The C/T 1858 polymorphism of *PTPN22* has been well documented as in association with several autoimmune diseases (Lee *et al.,* 2007). Previously, Canton *et al.,* (2005) showed an association of *PTPN22* C/T 1858 polymorphism with generalized vitiligo in Caucasian population (Canton *et al.,* 2005). In addition, two other studies from European population reported significant association of 1858C/T polymorphism with susceptibility to generalized vitiligo (Jin *et al.,* 2010; LaBerge, 2008). The present study shows that there is no association between *PTPN22* gene (C1858T) polymorphism with susceptibitlity to generalized vitiligo in Gujarat population (Table

2) and our results are in line with those of Alkhateeb *et al.,* (2010). There are several reports suggesting an association of C/T 1858 polymorphism with other autoimmune diseases (Bottini *et al.,* 2004; Orozco *et al.,* 2005; Begovich *et al.,* 2004; Zhernakova *et al.,* 2005). On the contrary, several reports also suggest a non association of *PTPN22* C/T 1858 polymorphism with autoimmune diseases (Hinks *et al.,* 2005; Martin *et al.,* 2005 Matesanz *et al.,* 2005). These contradictory reports may be because of the difference in ethnicity of the studied populations. However, in Indian population Ray *et al.* (2006) also reported a non association of *PTPN22* C/T 1858 polymorphism with rheumatoid arthritis suggesting that susceptible *PTPN22* 'T' allele is not prevalent in Indian population.

PTPN22 gene encodes a lymphoid-specific phosphatase (Lyp). Lyp is an intracellular protein tyrosine phosphatase (PTP) and physically bound through proline-rich motif to the SH3 domain of the Csk kinase, which is an important suppressor of kinases that mediate T-cell activation (Cohen and Dadi, 1999). The ability of Csk and Lyp to inhibit T-cell receptor signaling requires their physical association (Cloutier and Veillette, 1999). The C1858T substitution changes the amino acid from arginine (620R) to tryptophan (620W) at codon 620. This residue resides in the P1 proline-rich motif that is involved in binding to the SH3 domain of Csk (Cohen and Dadi, 1999). *In vitro* experiments have shown that the T-allele of *PTPN22* binds less efficiently to Csk than the C-allele does, suggesting that T-cells expressing the T-allele may be hyper responsive, and consequently, individuals carrying this allele may be prone to autoimmunity (Bottini *et al.,* 2004; Begovich *et al.,* 2004).

The exact genetic mechanism of autoimmune diseases is not well understood. Autoimmune diseases are also influenced by environmental factors in addition to genetic factors. The study could not attain statistical significance consequent to lower prevalence of 1858T allele in the studied population (Table 2). Interestingly, 1858T allele has been found to be absent in East Asians including Japanese (Ban *et al.,* 2005) Chinese (Begovich *et al.,* 2004) and South Asian Indians (Ray *et al.,* 2006). This was the first study to evaluate the association of the *PTPN22* 1858 C/T polymorphism with vitiligo in Gujarat population and this study has been published [Laddha NC,

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CHAPTER III B

GENETIC ASSOCIATION OF ANGIOTENSIN CONVERTING ENZYME (*ACE*) GENE POLYMORPHISM WITH GENERALIZED VITILIGO SUSCEPTIBILITY

3B.1 INTRODUCTION

Generalized vitiligo is a complex autoimmune disease in which patchy depigmentation of skin and hair results from loss of melanocytes from involved areas (Picardo and Taïeb, 2010) and is epidemiologically associated with several other autoimmune diseases (Alkhateeb *et al.*, 2003). Vitiligo, the most common pigmentary disorder, involves complex interaction of environmental and genetic factors that ultimately contribute to melanocyte destruction, resulting in the characteristic depigmented lesions. In the past few years, studies of the genetic epidemiology of vitiligo have led to the recognition that generalized vitiligo is part of a broader autoimmune disease diathesis.

Angiotensin converting enzyme (ACE), a key enzyme in the renin-angiotensin system, catalyzes the conversion of angiotensin I to angiotensin II in kidney through removal of two C-terminal residues. The ACE plays an important role in the physiology of the vasculature, blood pressure and inflammation, and it has also been found to be associated with several autoimmune disorders (Vuk-Pavlovic *et al.*, 1988, Scholzen *et al.*, 2003). The gene encoding *ACE* (or dipeptidyl carboxy peptidase 1: DCP1) is of 24 kb consisting of 26 exons, and is located on chromosome 17q23. An insertion/deletion (I/D) polymorphism of a 287-base pair repetitive sequence in intron 16 of the *ACE* gene (NCBI: AF118569; repeat region 14094–14381) has been associated with the development of vitiligo (Jin *et al.*, 2004; Deeba *et al.*, 2009; Tippisetty *et al.*, 2011). The insertion/deletion (I/D) polymorphism of the *ACE* gene accounts for a large proportion of the variability of serum ACE activity, D/D genotypes having the highest and I/I genotypes having the lowest ACE activity (Rigat *et al.*, 1990). In this study, the distribution of *ACE* I/D genotypes was investigated in generalized vitiligo patients and unaffected controls of Gujarat population to find the relationship between *ACE* I/D polymorphism and vitiligo.

3B.2 MATERIALS AND METHODS

3B.2.1 Study subjects:

The study group included 125 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 53 males and 72 females who referred

to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 156 ethnically, age and sex-matched unaffected individuals (68 males and 88 females) were included as controls in the study. None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of generalized vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 125)	(n = 156)
Average age (mean age ± SD)	30.34 ± 11.13 yrs	29.78 ± 11.25 yrs
Sex: Male	53 (42.40%)	68 (43.59%)
Female	72 (57.60%)	88 (56.41%)
Age of onset (mean age ± SD)	20.89 ± 12.61 yrs	NA
Duration of disease (mean ± SD)	7.42 ± 6.11 yrs	NA
Family history	17 (13.49%)	NA

3B.2.2 Genomic DNA preparation:

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

3B.2.3 Genotyping of *ACE* I/D (AF118569) polymorphism:

Polymerase chain reaction (PCR) method was used to genotype I/D polymorphism of *ACE* gene (Figure 1). The primers used for genotyping were:
5'CTGGAGACCACTCCCATCCTTTCT-3'(forward) and
5'GATGTGGCCATCACATTCGTCAGAT-3' (reverse).
The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 94°C for 10 minutes followed by 35 cycles of 94°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds.
The amplified products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.0% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

3B.2.4 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *ACE* gene I/D polymorphism in patients and controls by comparing the observed and expected frequencies of genotypes using chi-squared analysis. Distribution of the genotypes and allele frequencies of *ACE* I/D polymorphism for patients and control subjects were compared using the chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. *p*-values less than 0.05 were considered as statistically significant.

3B.3 RESULTS

3B.3.1 Analysis of association between *ACE* I/D (AF118569) polymorphism and susceptibility to vitiligo:

PCR for *ACE* I/D polymorphism yielded a 480 bp product corresponding to I allele and 193 bp products corresponding to D allele. The three genotypes identified by 2.0% agarose gel electrophoresis were: II homozygous, ID heterozygous and DD homozygous for I/D polymorphism of *ACE* gene (Figure 1).

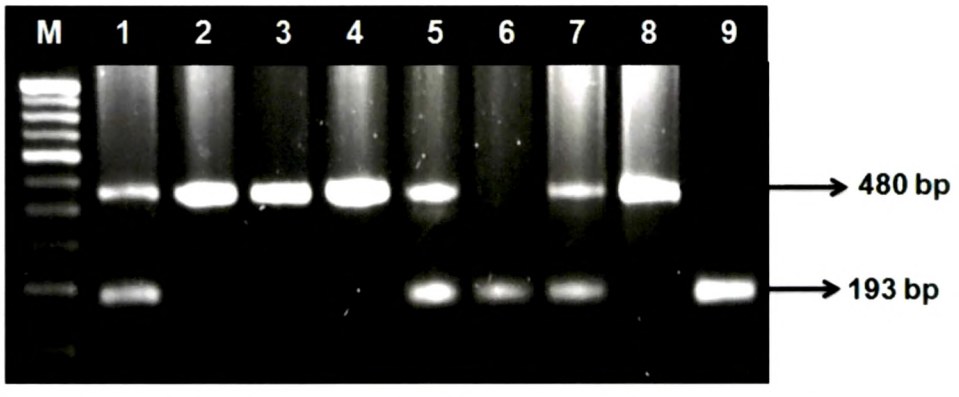

Figure 1. PCR analysis of *ACE* I/D polymorphism on 2.0% agarose gel electrophoresis: Lanes: 1, 5 & 7 show subjects with heterozygous ID genotypes; lanes: 2, 3, 4 & 8 show subjects with homozygous II genotypes; lanes: 6 &9 show subjects with homozygous DD genotypes; Lane: M shows a 100-bp ladder.

The genotype and allele frequencies of the *ACE* I/D polymorphism in 125 vitiligo patients and 156 controls are summarized in Table 2. No significant difference in the frequencies of I/I, I/D and D/D genotypes was detected between vitiligo patients and control subjects (p=0.459) using a 3×2 contingency table in a standard chi-squared test (Table 2), suggesting that there is no association of the *ACE* I/D polymorphism with vitiligo. In addition, the results indicate that the D allele is not significantly over-represented in the group of patients with vitiligo compared with controls (p=0.252) by using 2×2 contingency table in a standard chi-squared test (Table 2).

The observed allele frequencies of I and D were 0.56 and 0.44 respectively in controls; 0.61 and 0.39 respectively in vitiligo patients (Table 2). The distribution of

ACE I/D genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as patient groups (p=0.905 and p=0.964 respectively) (Table 2).

Table 2. Distribution of genotypes and alleles for the *ACE* I/D polymorphism in vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 125)	(n = 156)			
	II	46 (0.37)	51 (0.33)		0.964[a]	
ACE	ID	61 (0.49)	74 (0.47)	0.459	(P)	
I/D	DD	18 (0.14)	31 (0.20)		0.905[b]	
(AF118569)	Allele				(C)	1.219
	I	153 (0.61)	176 (0.56)	0.252		(0.8685-
	D	97 (0.39)	136 (0.44)			1.710)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.05$.

3B.4 DISCUSSION

Autoimmunity plays a major role in the pathogenesis of generalized vitiligo (Nordlund *et al.*, 2006; Kemp *et al.*, 2001). The frequent association of vitiligo with autoimmune disorders, (Ochi and DeGroot, 1969), the demonstration of autoantibodies to melanosomal proteins in the serum of patients with the disease, (Baharav *et al.*, 1996; Cui *et al.*, 1995; Kemp *et al.*, 1997; Song *et al.*, 1994) and evidence that vitiligo autoantibodies can destroy melanocytes *in vitro*, (Norris, 1998) and *in vivo* (Gilhar *et al.*, 1995) support this theory.

ACE and its related substrates or products are known to have various functions in the immune system (Vuk-Pavlovic *et al.*, 1988) and in the inflammatory response.

However, its underlying pathogenic mechanism in autoimmune diseases is yet to be understood.

ACE is capable of degrading the substance P (SP) and other peptide mediators (Scholzen *et al.*, 2003). In the skin, neuropeptides such as SP are released from sensory nerves in response to noxious stimuli like chemical and mechanical injury. SP may induce or augment inflammatory responses such as plasma extravasation, leukocyte activation, cytokine production, and mast cell activation (Scholzen *et al.*, 1998).

ACE catalyzes the conversion of angiotensin I to angiotensin II which is a potent mediator of oxidative stress and stimulates the release of cytokines and the expression of leukocyte adhesion molecules that mediate vessel wall inflammation. Inflammatory cells release enzymes (including ACE) that generate angiotensin II which in turn can induce cytokines such as TNF-α and IFN-γ (Dzau, 2001). Our recent studies found increased transcript and protein levels of TNF-α and IFN-γ in vitiligo patients (unpublished data). These increased cytokines may modulate the microenvironment which in turn can signal for apoptosis of melanocytes as observed in vitiligo patients. Previous investigations have shown that elevated levels of ACE have been associated with autoimmune diseases (Czernobilsky *et al.*, 1985; Papadopoulos *et al.*, 2000) and that the D allele of *ACE* I/D polymorphism confers susceptibility to autoimmune disorders (Papadopoulos *et al.*, 2000) and vitiligo (Jin *et al.*, 2004).

Most of the studies reported significant association of *ACE* I/D polymorphism with vitiligo, although some yielded only marginal significance and some were not replicated by subsequent studies. Earlier, the D allele of I/D polymorphism of the *ACE* gene was reported to confer susceptibility to vitiligo in Korean population (Jin *et al.*, 2004). A recent study from South India revealed a significant association of DD genotype with vitiligo susceptibility in patients with a family history of vitiligo (Tippisetty *et al.*, 2011). Deeba *et al.* (2009) from South India also reported significant association of *ACE* I/D polymorphism with vitiligo (Deeba *et al.*, 2009). However, the current study suggests that the polymorphic variant D allele is not associated with vitiligo susceptibility (Table 2). This may be because of the differences in ethnicity of the studied populations. Our results are comparable to the

previous report in which genotype frequencies for *ACE* I/D polymorphism were not significantly different between controls and generalized vitiligo patient population (Akhtar *et al.*, 2005). Our results on the distribution rate of I/D > I/I > D/D in the control population are in accordance with the previously reported distribution rate of I/D for Chinese (Lee *et al.*, 1994), Japanese (Ishigami *et al.*, 1995) and Korean population (Jin *et al.*, 2004). Moreover, a study from Turkey did not find significant association of *ACE* I/D polymorphism with vitiligo susceptibility (Pehlivan *et al.*, 2009).

The molecular basis of autoimmune diseases is not yet well understood. Autoimmune diseases are also influenced by environmental factors in addition to genetic factors (Zhang *et al.*, 2004). The study could not attain statistical significance consequent to lower prevalence of D allele in the studied population. This was the first study to evaluate the association of the *ACE* I/D polymorphism with vitiligo in Gujarat population where prevalence of vitiligo is alarmingly high and we have published these results [Dwivedi M, Laddha NC, Shajil EM, Shah BJ and Begum R (2008). The ACE gene I/D polymorphism is not associated with generalized vitiligo susceptibility in Gujarat population. Pigment Cell Melanoma Res. 21: 407-408].

In conclusion, the *ACE* gene I/D polymorphism does not appear to play a role in the development of generalized vitiligo and hence it may not be a genetic risk factor for vitiligo susceptibility in Gujarat population.

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CHAPTER III C

MANNAN BINDING LECTIN-2 (*MBL2*) GENE STRUCTURAL AND PROMOTER POLYMORPHISMS IN GENERALIZED VITILIGO

3C.1 INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder in which progressive, patchy loss of pigmentation from skin, overlying hair, and oral mucosa results from autoimmune loss of melanocytes from the involved areas (Nordlund *et al.*, 2006). It affects 0.5–1% of the world population (Taieb and Picardo, 2007). In India the incidence of vitiligo is found to be 0.5-2.5% (Handa and Kaur, 1999) where as Gujarat and Rajasthan states have the highest prevalence of ~8.8% (Valia and Dutta, 1996). The etiology of vitiligo is still unknown, but it is hypothesized to be of autoimmune origin due to its frequent association with several autoimmune diseases, the presence of circulating autoantibodies and autoreactive T-cells in the sera of vitiligo patients compared to unaffected individuals (Shajil *et al.*, 2006; Kemp *et al.*, 2001).

Various factors such as oxidative stress, genetic factors, neural factors, toxic metabolites and lack of melanocyte growth factors might contribute for precipitating the disease in susceptible people (Njoo and Westerhof, 2001). The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Nordlund, 1997). It has been found that about 20% of vitiligo patients have at least one first-degree relative affected (Nath *et al.*, 1994). Previous studies showed that 22% of Gujarat vitiliginous patients exhibit positive family history and 14% patients have at least one first-degree relative affected (Shajil *et al.*, 2006). The disease does not follow the simple Mendelian inheritance pattern and its mode of heredity suggests that it is a polygenic disease.

Furthermore, several genes are involved in regulating immunity have been associated with susceptibility to vitiligo including allelic variants in the cytotoxic T -lymphocyte antigen-4 gene (*CTLA4*) (Blomhoff *et al.*, 2005; Kemp *et al.*, 1999; McCormack *et al.*, 2001), the autoimmune susceptibility loci *AIS1, AIS2, AIS3* and *SLEV1* (Alkhateeb *et al.*, 2001; Fain *et al.*, 2003; Spritz *et al.*, 2004), the autoimmune regulator (*AIRE*) gene (Nagamine *et al.*, 1997), NACHT leucine-rich-repeat protein 1 (*NALP1*- maps at *SLEV1* sucsceptibility locus) (Jin *et al.*, 2007a,b), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) (Laberge *et al.*, 2008a,b ; Vang *et al.*,

2007) and certain human leukocyte antigen specificities of the major histocompatibility complex (Le Poole *et al.*, 2008, Fain *et al.*, 2006).

Mannan-binding lectin (MBL) [synonyms: mannose binding lectin (MBL), mannose binding protein (MBP), mannan binding protein (MBP)] is a liver-derived calcium dependent serum protein, which plays an important role in innate immune defense. MBL is a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure resembling C1q of the complement pathway. MBL binds to cell surface ligands such as mannose, N-acetylglucosamine residues, which are expressed on a wide range of microorganisms. MBL binds to carbohydrate moieties on microorganisms and thus leads to activation of the complement system via the lectin pathway (Neth *et al.*, 2000). In addition to complement activation, the protein has several distinct functions including promotion of complement-independent opsono-phagocytosis, modulation of inflammation and promotion of apoptosis (Turner, 2003). Role of the MBL pathway in complement activation and in the clearance of apoptotic cells suggests that genetic variability in *MBL* may be involved in the pathogenesis of autoimmune diseases (Turner and Hamvas, 2000). Possible role of MBL in vitiligo pathogenesis could be due to defective clearance of apoptotic cells which may substantially contribute to the triggering of autoimmune responses (Werth *et al.*, 2002). Also, MBL is involved in inflammatory response, regulation of pro-inflammatory cytokines, controlling adhesion molecules on inflammatory cells and immune complex removal. Thus these studies emphasize the importance of MBL in cutaneous autoimmunity (Jack *et al.*, 2001).

The functional *MBL2* gene is located on chromosome 10 (q11.2-q21) and comprises of four exons. Exon 1 encodes the signal peptide, a cysteine rich region and part of the glycine-rich collagenous region. Three functional single-nucleotide polymorphisms (SNPs) in exon 1 of *MBL2* gene have been reported: codon 54 (G**G**C→**GA**C; designated B allele), codon 57 (G**G**A→**GA**A; designated C allele), and codon 52 (**C**GT→**T**GT; designated D allele) (Sumiya *et al.*, 1991; Lipscombe *et al.*, 1992; Hansen and Holmskov, 1998). These variant alleles in the collagen like domain cause structural changes in MBL and will lead to its low plasma levels and thus results in plasma concentrations varying by more than 1000-fold within the population (5 ng/ml to 5 μg/ml) (Garred *et al.*, 2003; Thiel, 2007). The promoter region of the *MBL2* gene

contains a number of regulatory elements, which affect transcription of the gene and hence polymorphisms in the promoter region, thus affecting the plasma MBL concentration directly. Besides these, exon 1 variant alleles, SNPs at promoters -550 (G/C; allele L) and -221 (G/C; allele X) have also been associated with low plasma levels of MBL (Madsen *et al.*, 1995). Previously, an association between *MBL2* gene codon 54 (allele B) polymorphism and susceptibility to vitiligo has been described in Turkish population (Onay *et al.*, 2007).

The aim of this study was to investigate the association of *MBL2* structural (codon 52, codon 54 and codon 57) and promoter (-221) polymorphisms with vitiligo susceptibility in Gujarat population.

3C.2 MATERIALS AND METHODS

3C.2.1 Study Subjects:

The study group included 92 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 29 males and 63 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). Of which 62 patients and 72 age matched controls were genotyped for codon 52 (allele D), 84 patients and 81 controls were genotyped for codon 54 (allele B), and 68 patients and 72 controls were genotyped for codon 57 (allele C). For genotyping of -221 promoter polymorphism, 92 patients and 93 control subjects were used. The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 94 ethnically sex-matched unaffected individuals (61 males and 34 females) were included as controls in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of generalized vitiligo patients and unaffected controls.

	Generalized Vitiligo (GV) Patients	**Controls**
	(n = 92)	(n = 94)
Average age (mean age ± SD)	28.37 ± 1.740 yrs	27.28 ± 0.841 yrs
Sex: Male	29 (31.52%)	61 (64.89%)
Female	63 (68.48%)	34 (36.17%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Family history	12 (13.04)	

3C.2.2 Genomic DNA preparation:

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

3C.2.3 Genotyping of *MBL2* structural and promoter polymorphisms:

The exon 1 structural and promoter polymorphisms were genotyped by polymerase chain reaction (PCR)-heteroduplex analysis technique. This technique involves the use of a universal heteroduplex generator (UHG), a synthetic DNA molecule based on the target genomic sequence (Jack *et al.*, 1997). Genomic DNA-polymerase chain reaction (PCR) product was combined with UHG-PCR product and the two were allowed to anneal to produce characteristic heteroduplexes for different alleles. These

heteroduplexes displayed different electrophoretic mobilities on a polyacrylamide gel enabling identification of a subject's genotype (Jack *et al.*, 1997).

3C.2.4 PCR amplification of genomic DNA and UHG:

Two separate polymerase chain reactions (PCRs) were performed on genomic DNA samples to amplify exon 1 and -221 (X/Y) promoter regions by using forward (5'CCAACACGTACCTGGTTCC3') and reverse (5'CTGTGACCTGTGAGGATG-C3') primers for exon 1 and forward (5'AGGCATAAGCCAGCTGGCAAT3') and (5'CTAAGGAGGGGTTCATCTG3') reverse primers for -221 (X/Y) promoter region.

All primers were synthesized from MWG- Biotech, Germany. The PCR was performed in a 20 µl system containing 50 ng genomic DNA, 0.25mM dNTPs, 20 pmoles of each primer, 2mM $MgCl_2$, PCR gold buffer and 1.5U Amplitaq Gold DNA polymerase (Applied Biosystems, USA). PCR amplification was carried out in a thermal cycler (ABI 9700) with the following conditions: initial denaturation at 95^0C for 3 min followed by denaturation at 95^0C for 30 sec, annealing at 56^0C for 30 sec, extension at 72^0C for 30 sec and final extension at 72^0C for 7 min. For the synthesis of each UHG, same PCR conditions were used with each 50 ng UHG template (template size of 133 bp for exon 1 and 111 bp for -221 promoter region) which were commercially synthesized UHG (MWG- Biotech, Germany), specifically designed to detect the exon 1 and promoter regions of *MBL2*. PCR products were analyzed on a 2% agarose gel prior to heteroduplexing and confirmed with 100 bp DNA ladder (MBI Fermentas, Canada). Confirmation of specific amplification of genomic and UHG DNA using 2% agarose gel electrophoresis enabled comparison of the relative amounts of DNA obtained from each sample reaction against those from the UHG reaction.

3C.2.5 Heteroduplex analysis:

A mixture of approximately equal amounts of genomic and UHG-PCR products were combined prior to heating to 95^0C for 5 minutes and then allowed to cool at room temperature over 2 hours to enable greater specific annealing. The PCR mix was spun

briefly and the appropriate volume of 6x loading dye was added to each mix. Heteroduplexes thus formed were checked by 20% polyacrylamide gel electrophoresis followed by ethidium bromide staining (Figures 1 & 2).

On hetroduplexing homozygotes developed two heteroduplex bands and heterozygotes generated four heteroduplex bands (Figurel & 2). Exception was WT/R52C where only three bands were generated due to co-migration of two heteroduplexes as a single upper band (Jack *et al.*, 1997) (WT designating one wild-type allele and R52C, G54D, and G57E, indicating an allele with a mutation at codons: 52, 54 and 57 respectively) (Figure 1). Interpretation of band patterns was done manually with the aid of known samples used as reference grid. The genotype WT/G57E generating four bands spanning the whole gel was taken as reference, and was included in each run. The position of all the other bands could be judged using this sample as reference.

3C.2.6 Statistical analysis:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for the polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis. The distribution of the *MBL2* genotypes and allele frequencies for patients and control subjects were compared using the chi-squared test with 3×2 and 2×2 contingency tables respectively using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California, USA. The results were supported by odds ratios (OR) and 95% confidence intervals (CI) when appropriate. *p*-values less than 0.0125 were considered as statistically significant due to Bonferroni's correction for multiple testing.

3C.3 RESULTS

3C.3.1 Analysis of association between *MBL2* exon 1 structural polymorphisms and susceptibility to vitiligo:

Codon 52, codon 54 and codon 57 polymorphisms of *MBL2* exon 1 were not found to be associated with generalized vitiligo patients (p=0.019 for codon 52, p=0.373 for

codon 54, p=0.855 for codon 57) when genotypes were compared with chi-square test-3x2 contingency table (Table 2). Also, there was no significant difference in allele frequencies of codon 52, codon 54 and codon 57 between patients and controls when compared with 2x2 contingency table (p=0.020 for D allele, p=0.378 for B allele and p=0.858 for C allele) (Table 2). Both patients and control population were found to be in Hardy-Weinberg equilibrium for all the three exon 1 codon polymorphisms (p<1.000 and p<0.932 for codon 52 polymorphism respectively; p=0.869 and p=0.650 for codon 54 polymorphism respectively and p=0.932 and p=0.909 for codon 57 polymorphism respectively) (Table 2). The genotypes for *MBL2* structural polymorphisms were confirmed by running heteroduplexes on 20% polyacrylamide gel (Figure 1).

The association study for exon 1 polymorphisms was also done by A/O genotyping where all the three codon polymorphisms were placed in one group designated as 'O' allele and wild type as 'A' allele. The A/O genotypes were compared between controls and patients using chi-square-3x2 contingency table. Interestingly, there was no significant association found between the A/O genotype and generalized vitiligo (p=0.999) (Table 1). Furthermore, 'O' allele was also not significantly associated with any of the population when compared using 2x2 contingency table (p=0.968) (Table 1).

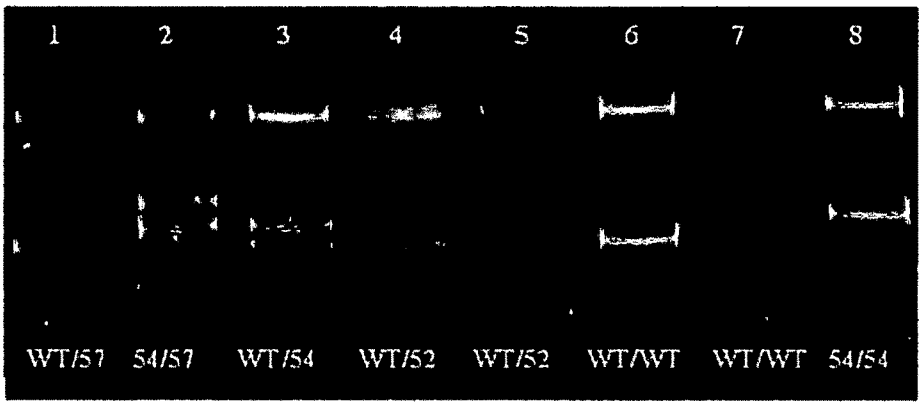

Figure 1. PCR-heteroduplex analysis of *MBL2* exon 1 (codon 52, codon 54 and codon 57) polymorphisms on 20% polyacrylamide gel electrophoresis: lane 1 shows heterozygous WT/57; lane 2 shows heterozygous 54/57; lane 3 shows heterozygous WT/54; lanes 4, 5 show heterozygous WT/52; lanes 6, 7 show homozygous WT/WT and lane 8 shows homozygous 54/54 individual.

Table 2. Association studies for mannan-binding lectin gene (*MBL2*) exon 1 polymorphisms in Gujarat patients with generalized vitiligo.

SNP	Genotype or allele	Vitiligo Patients	Controls	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n =62)	(n =72)			
	TT	00	00		1.0	
rs5030737	CT	00	06	0.0192	(P)	0.08
Codon 52	CC	62	65		0.932	(0.004-
(allele D)	Allele				(C)	1.462)
	T	00	08	0.007		
	C	124	136	(#0.02)		
	Genotype	(n = 84)	(n = 81)			
	AA	01	00			
rs1800450	AG	21	15	0.373	0.869	1.353
Codon 54	GG	62	65		(P)	(0.694-
(allele B)	Allele				0.650	2.639)
	A	23	17	0.378	(C)	
	G	145	145			
	Genotype	(n = 68)	(n = 72)			
	AA	00	00			
rs1800451	AG	06	07	0.855	0.932	0.899
Codon 57	GG	62	65		(P)	(0.286-
(allele C)	Allele				0.909	2.823)
	A	06	07	0.858	(C)	
	G	130	137			
	Genotype	(n =90)	(n = 94)			
	OO	01	01			
Genotype	AO	27	28	0.999	0.579	0.989
A/O†	AA	62	65		(P)	(0.566-
	Allele				0.563	1.726)
	A	151	158	0.968	(C)	
	O	29	30			

'n' represents number of Patients/ Controls,
HWE refers to Hardy-Weinberg Equilibrium,
† A is the designation for wild-type alleles, and O is the common designation for the variant alleles B, C, and D;
(P) refers to Patients and (C) refers to Controls.
Yate's corrected *p* value.

3C.3.2 Analysis of association between *MBL2* -221 promoter polymorphism and susceptibility to vitiligo:

In addition, we have also genotyped the -221 (X/Y) promoter polymorphism of *MBL2* gene in 92 patients and 93 controls. The genotypes for -221 promoter polymorphism were confirmed by running heteroduplexes on 20% polyacrylamide gel (Figure 2). There was no significant association found between -221 promoter polymorphism and the risk of vitiligo when genotype frequencies of patients and controls were compared with chi-square test-3x2 contingency table (p=0.889) (Table 3). Also, the allele frequencies of patients and controls for -221 (X/Y) promoter polymorphism did not show significant difference when compared with 2x2 contingency table (p=0.765) (Table 3). However, both controls and patient population were in accordance with Hardy-Weinberg equilibrium (p=0.120 and p=0.370 respectively) (Table 3).

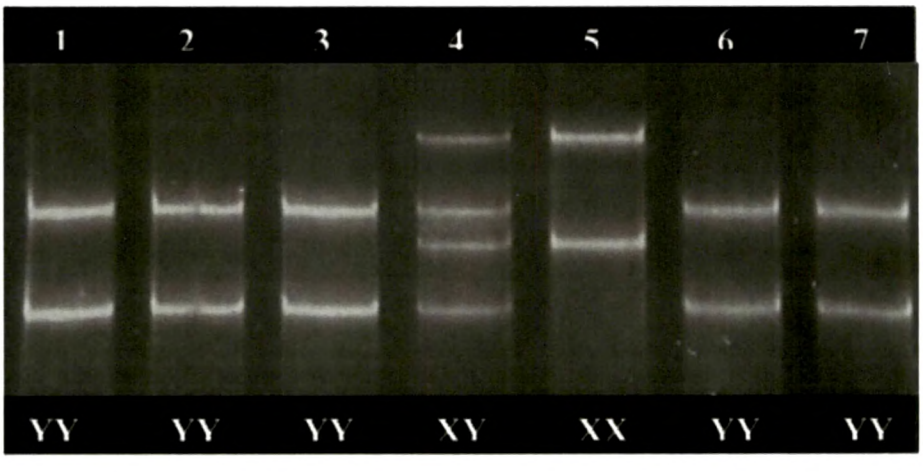

Figure 2. PCR-heteroduplex analysis of *MBL2* -221 (X/Y) promoter polymorphism on 20% polyacrylamide gel electrophoresis: lanes 1, 2, 3, 6, 7 show homozygous YY; lane 4 shows heterozygous XY and lane 5 shows homozygous XX individual.

Table 3. Distribution of alleles and genotypes for the -221 (X/Y; rs7096206) promoter polymorphism of *MBL2* gene in generalized vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 92)	(n = 93)			
	XX	08 (0.09)	10 (0.11)			
MBL2	XY	29 (0.31)	28 (0.30)	0.889	0.370[a]	
-221	YY	55 (0.60)	55 (0.59)		(P)	0.9308
(X/Y;	Allele					(0.5817-
rs7096206)	X	45 (0.24)	48 (0.26)	0.765	0.120[b]	1.489)
	Y	139 (0.76)	138(0.74)		(C)	

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.05$.

3C.3.3 Haplotype analysis:

The promoter and structural genotypes of patients and controls were combined to construct haplotypes and to find whether there was any haplotype associated with vitiligo susceptibility. Interestingly, no haplotype was found to be associated with generalized vitiligo when compared with chi-square test (p=0.962) (Table 4).

Moreover, the haplotypes were distributed to represent MBL levels as: high (YA/YA, YA/XA), medium (XA/XA, YA/YO) and low (XA/YO, YO/YO) in both controls and patients. However, no significant difference was found in the distribution of high, medium and low haplotypes when compared with 2x2 contingency table (p=0.838) (Table 4).

Table 4. Distribution of mannan-binding lectin (*MBL2*) gene combined (structural & promoter) genotypes among generalized vitiligo patients and controls.

Combined Genotype (Structural + Promoter)	Vitiligo Patients (n=90)	Controls (n=88)	*p* for Association
YA/YA	33 (36.67%)	34 (38.63%)	0.962
YA/XA	21 (23.34%)	16 (18.18%)	
YA/YO	20 (22.22%)	19 (19.19%)	
XA/XA	08 (8.89%)	09 (10.22%)	
XA/YO	07 (7.78%)	09 (10.10%)	
YO/YO	01 (1.11%)	01 (1.10%)	
MBL High (YA/YA, YA/XA)	54 (60.00%)	50 (56.80%)	0.838
MBL Medium (XA/XA, YA/YO)	28 (32.20%)	28 (31.20)	
MBL Low (XA/YO, YO/YO)	08 (8.89%)	10 (11.36%)	

3C.4 DISCUSSION

Vitiligo susceptibility is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo since it shows frequent association with other autoimmune disorders such as rheumatoid arthritis, Graves' disease etc. (Ochi *et al.*, 1969). One of the important regulators of innate immunity is *MBL2* which is involved in complement activation and clearance of apoptotic cells suggesting its importance in pathogenesis of autoimmune diseases. We therefore, selected *MBL2* as a candidate gene to study vitiligo susceptibility in Gujarat population and our results suggest that there is no association of *MBL2* structural and promoter polymorphisms with vitiligo susceptibility.

MBL is a pattern-recognition molecule of the innate immune system and primarily recognizes specific sugar groups on the surface of microorganisms, enabling it to distinguish self from non-self. The three structural variants in exon 1 of *MBL2* gene are associated with decreased plasma levels of MBL compared with homozygotes of the wild type gene (Wallis *et al.*, 2002). The structural alterations in the MBL due to three coding polymorphisms disrupt interactions of MBL with the associated serine proteases, there by greatly diminishing complement-activating ability (Wallis *et al.*, 2002; Yokota *et al.*, 1995).

The MBL concentration is also regulated by two additional promoter polymorphisms namely: -550 (G → C; allele L) and -221 (G → C; allele X) which directly affect the expression of the *MBL* gene. Since MBL is one of the proteins shown to be involved in the clearance of apoptotic cells, Werth *et al*, suggested that any defect in the clearance of apoptotic cells may result in aggravation of autoimmune responses (Werth *et al.*, 2002). In addition, MBL is also involved in inflammatory response, regulation of pro-inflammatory cytokines, controlling adhesion molecules on inflammatory cells and immune complex removal; thus emphasizing its importance in cutaneous autoimmunity (Jack *et al.*, 2001). Reports from Schallreuter *et al.*, showed a perturbed calcium homeostasis in vitiliginous melanocyte and keratinocyte cell cultures (Schallreuter *et al.*, 1996; Schallreuter *et al.*, 1988) suggesting that vitiligo

patients carrying *MBL* functional and promoter polymorphisms may have defective calcium uptake resulting in low MBL concentration.

MBL2 gene polymorphisms have been associated with increased risk of autoimmune diseases such as systemic lupus erythematosus (SLE) in both Caucasoid and Chinese populations (Davies *et al.*, 1995; Ip *et al.*, 1998) cutaneous lupus erythematosus and dermatomyositis (Werth *et al.*, 2002). Senaldi *et al.* (1995) suggested that patients with SLE had a higher frequency of low levels of MBL than controls. Wang *et al.* (2001) reported that a codon 54 (allele B) of the *MBL2* gene might be associated with genetic susceptibility to Sjögren's syndrome (SJS). Thus most of the studies show that MBL deficiency is associated with poorer outcome as judged by clinical, inflammatory and radiographic indices (Graudal *et al.*, 1998; Graudal *et al.*, 2000). However, evaluation of *MBL2* coding mutations in Japanese population found no association with SLE and rheumatoid arthritis (RA) (Horiuchi *et al.*, 2000). Graudal *et al.* (1998) suggested that there is a greater prevalence of MBL deficiency in patients with RA but other studies refute this assertion (Kilpatrick *et al.*, 1997). Rector *et al.* (2001) found no association between structural polymorphisms in exon 1 of *MBL2* gene and susceptibility to Crohn's disease.

Since vitiligo is hypothesized to be of autoimmune origin, it becomes relevant to screen vitiligo patients for the presence of *MBL2* structural and promoter variants which may predispose an individual for vitiligo. Earlier, an association of codon 54 (allele B) with vitiligo was observed in Turkish population where only two codons 54 and 57 were genotyped with smaller sample size (Onay *et al.*, 2007). Hence, it was pertinent to study all such important polymorphisms of *MBL2* with an adequate sample size and we found there was no association between vitiligo and *MBL2* structural and promoter polymorphisms. The positive association of codon 54 polymorphism and susceptibility to vitiligo described by Onay *et al.* (2007) may be due to differences in the ethnicity of the studied populations.

This is the first report that shows non-association of structural and promoter polymorphisms of *MBL2* gene with generalized vitiligo and our results are in line with those of Birlea *et al.* (2011) suggesting the non-association of generalized vitiligo with codon 54 (rs1800450) or with any of the studied 15 SNPs spanning the *MBL2* region. This study has been published [Dwivedi M, Gupta K, Gulla KC,

Laddha NC, Hajela K and Begum R; Lack of genetic association of promoter and structural variants of Mannan-binding lectin (*MBL2)* gene with susceptibility to generalized vitiligo. *Brit. J Dermatol.* (2009) **161,** 63-69].

In conclusion, it can be considered that the structural and promoter polymorphisms in the *MBL2* gene may not confer a role in generalized vitiligo susceptibility of Gujarat population.

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CHAPTER IV

INTERFERON-γ (*IFNG*) GENE INTRON 1 POLYMORPHISMS AND THEIR GENOTYPE-PHENOTYPE CORRELATION WITH GENERALIZED VITILIGO SUSCEPTIBILITY

4.1 INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder in which progressive, patchy loss of pigmentation from the skin, overlying hair, and oral mucosa occurs due to autoimmune loss of melanocytes from the involved areas (Nordlund *et al.*, 2006). It is frequently associated with a positive family history and affects approximately 0.5-1% of the world population (Taieb and Picardo, 2007). In India, the incidence of vitiligo is found to be 0.5-2.5% (Handa and Kaur, 1999), where as Gujarat and Rajasthan states have the highest prevalence i.e., ~8.8% (Valia and Dutta, 1996).
The etiology of the disease is still unknown, but the loss of melanocytes can be explained due to autoimmune mechanism, or by autocytotoxic mechanism, or by neural dysfunction (Bystryn, 1989). The autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity (Shajil *et al.*, 2006; Kemp *et al.*, 2001). The autoimmune hypothesis gains further support from immunotherapy studies of melanoma patients (Rosenberg, 1997). Twenty six percent of melanoma patients responded to interleukin (IL)-2 based immunotherapy, developed vitiligo. This finding suggests that antimelanotic T cells which might be responsible for melanoma regression may also be linked to the destruction of normal melanocytes in vitiligo (Zeff *et al.*, 1997). Indeed, it was clearly demonstrated that cytotoxic T cells (CTL) generated from melanoma tissue also recognize differentiation antigens expressed by normal melanocytes (Anichini *et al.*, 1993).

Vitiligo is a polygenic disease and several candidate genes including the major histocompatibility complex (*MHC*), angiotensin converting enzyme (*ACE*), catalase (*CAT*), cytotoxic T-lymphocyte associated antigen 4 (*CTLA4*), catechol o-methyl transferase (*COMT*), estrogen receptor (*ESR*), mannose-binding lectin 2 (*MBL2*) and protein tyrosine phosphatase non-receptor type-22 (*PTPN22*) that regulate immune mechanisms have been tested for genetic association with generalized vitiligo (Spritz *et al.*, 2007; Spritz *et al.*, 2008).

Cytokines are small immune-regulatory molecules which can generate an inappropriate immune response due to their imbalance. Morreti *et al.* (2003) have shown cytokine imbalance in skin of vitiligo patients suggesting their role in autoimmunity. Interferons are a small group of cytokines that include alpha, beta and

gamma interferons (IFN-α, β, γ). The principal sources of IFNγ are helper T cells and natural killer cells, although many other cells can produce it including keratinocytes. Interferon-γ (type II interferon) is a single copy gene located on human chromosome 12q24 spanning approximately 5.4 kb, consisting of four exons and three introns (Gray and Goeddel, 1982; Naylor *et al.*, 1983). It encodes acid-labile protein of 166 amino acids which has antiviral, immune-regulatory and anti-tumour properties (Schroder *et al.*, 2004). It alters transcription of around thirty genes involved in a variety of physiological and cellular responses. IFNγ binding to IFNGR1 & IFNGR2 (interferon gamma receptors) activates the JAK-STAT pathway by phosphorylation of JAK and thereby activating STAT transcription factor (Horvath, 2004). In addition, IFNγ activates antigen presenting cells (APCs) and promotes Th1 differentiation by up regulating the transcription factor and inhibiting the development of Th2 cells (Oriss *et al.*, 1997).

IFNγ is a pleiotropic cytokine and is a key regulator of immune system. Apart from host defense, IFNγ may also contribute to autoimmune pathology. Although IFNγ production was shown to be disease-limiting in autoimmune models such as murine experimental allergic encephalomyelitis (EAE) (Espejo *et al.*, 2001) it may contribute to autoimmune nephritis (Heremans *et al.*, 1978). In humans, IFNγ is implicated in pathology of diseases such as systemic lupus erythematosus (Lee *et al.*, 2001), multiple sclerosis (Panitch *et al.*, 1987), and insulin-dependent diabetes mellitus (Wang *et al.*, 1997). *IFNG* mRNAs have been detected in normal human melanocytes (Satomi *et al.*, 2002). IFNγ is suggested to be important in enhancing biological defense activities against oxidative stress (Lu *et al.*, 2002) and may cause induction of antimelanocyte autoantibodies or activation of cytotoxic T cells (SeÈkin *et al.*, 2004; Miller *et al.*, 2002).

It has been suggested that melanocyte death is mediated by apoptosis in the context of autoimmunity, and cytokines such as IFNγ and TNFα can initiate apoptosis (Huang *et al.*, 2002). Additionally, IFNγ and TNFα induce the expression of intercellular adhesion molecule-1 (*ICAM1*) on the cell-surface of melanocytes (Yohn *et al.*, 1990). Increased expression of this adhesion molecule on the melanocytes enhances T cell-melanocyte attachment in the skin and may play a role in the destruction of melanocytes in vitiligo (Al Badri, 1993).

These reports signify that the host ability to produce cytokine such as IFNγ may play a crucial role in vitiligo susceptibility. Therefore, it is possible that individuals who naturally produce higher levels of this cytokine will exhibit different susceptibility, or severity towards vitiligo.

The *IFNG* coding region is invariant, with no reported polymorphisms (Hayden *et al.*, 1997). However, there are two well-known single-nucleotide polymorphisms in the *IFNG* gene non-coding region. A CA repeat microsatellite sequence in the non-coding region of the first intron is polymorphic. Allele 2, with 12 CA repeats is associated with constitutively high IFNγ production *in vitro*. In addition, a single nucleotide A/T polymorphism at the 5' end of the CA repeat region in the first intron of the *IFNG* gene (+874 A/T polymorphism; rs2430561) has been correlated with the presence or absence of the microsatellite allele 2 (Pravica *et al.*, 1999). Also, the presence of *IFNG* (+874 T^{hi}/A^{lo}) polymorphism creates a putative NF-κB binding site in intron1 and shows preferential binding to the T allele and correlates with high IFNγ producer phenotype (Pravica *et al.*, 2000).

In the present study, we have made an attempt to understand the role of IFNγ in vitiligo pathogenesis. Hence, the objectives of this study were:

i.) To determine whether the intron 1 polymorphisms of *IFNG* [+874A/T (rs2430561) and 5' end CA microsatellite (rs3138557)] are associated with vitiligo susceptibility.

ii.) To measure and compare *IFNG* and *ICAM1* transcripts and serum IFNγ levels in patients with vitiligo and in unaffected controls.

iii.) To correlate *IFNG* polymorphisms/levels with onset and progression of the disease.

4.2 MATERIALS AND METHODS

4.2.1 Study Subjects:

The study group included 517 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 222 males and 295 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of

depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 881 ethnically sex-matched unaffected individuals (397 males and 484 females) were included in this study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of generalized vitiligo patients and unaffected controls.

	Generalized Vitiligo (GV) Patients	**Controls**
	(n = 517)	(n = 881)
Average age (mean age ± SD)	31.34 ± 12.18 yrs	28.04 ± 13.16 yrs
Sex: Male	222 (42.94%)	397 (45.06%)
Female	295 (57.06%)	484 (54.94%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Type of vitiligo		
Active vitiligo	382 (73.89%)	NA
Stable vitiligo	135 (26.11%)	NA
Family history	65 (12.57%)	NA

4.2.2 Blood collection and DNA extraction:

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically,

quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

4.2.3 Genotyping of +874 A/T SNP of *IFNG* by ARMS-PCR:

IFNG +874A/T genotyping was done using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method as described before (Faul *et al.*, 2007). DNA was amplified in two different PCR reactions with a generic antisense primer and one of the two allele specific sense primers (Table 2). To assess the success of PCR amplification in both the reactions, an internal control of 426 bp was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone (*HGH*) (Table 2). The reaction mixture of the total volume of 15.1 µL included 5 µL (100 ng) of genomic DNA, 5.0 µL nuclease-free H_2O, 1.5 µL 10x PCR buffer, 0.3 µL 8 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM allele-specific and common primers (MWG Biotech, India), 0.3 µL of 2 µM control primers (*HGH*), 1.5 µL Mg^{2+} and 0.6 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India).

Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 1 minute followed by 10 cycles of 95°C for 15 seconds, 62°C for 50 seconds, and 72°C for 40 seconds, then 20 cycles of 95°C for 20 seconds, 56°C for 50 seconds, and 72°C for 50 seconds.

The amplified products were separated by electrophoresis on 2.0% agarose gel and stained with ethidium bromide. The gel was visualized under a UV transilluminator with a 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) and photographed. Two amplicons were available for each sample (one each specific for T or A allele of the *IFNG* gene). More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently and also confirmed by sequencing).

4.2.4 Genotyping of CA microsatellite (rs3138557) of *IFNG* by high resolution melt (HRM) curve analysis:

IFNG CA microsatellite (rs3138557) was genotyped by High Resolution Melt (HRM) curve analysis using LightCycler® 480 Real-Time PCR protocol. Real-time PCR was performed in 20 µl volume using LightCycler®480 HRM Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 15 s, primer dependent annealing for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a high resolution melting curve analysis was carried out on the product formed as per the manufacturer's instructions. DNA sequencing was carried out for samples from each representative groups obtained by HRM analysis.

4.2.5 Determination of *IFNG, ICAM1* and *GAPDH* mRNA expression:

4.2.5.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

4.2.5.2 Real-time PCR:

The expression of *IFNG, ICAM1* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed

in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (94°C for 15 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 8). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

Table 2. Primers used for *IFNG* +874A/T (rs2430561) and CA microsatellite (rs3138557) polymorphisms genotyping and gene expression analyses.

Gene/SNP Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)
(rs2430561)			
IFNG A/T F*	TCAACAAAGCTGATACTCCA	62	265
IFNG A R*	TTCTTACAA CACAAAATCAAATCA		
IFNG T R*	TTCTTACAACACAAAATCAAATCT		
HGH F	CCTTCCCAACCATTCCCTTA	56	428
HGH R	TCACGGATTTCTGTTGTGTTTC		
(rs3138557)			
IFNG CA F	GGTTTCTATTACATCTACTGTGC	56	149
IFNG CA R	CAGACATTCACAATTGATTTTATTC		
IFNG expression F	TTGGAAAGAGGAGAGTGACAG	65	212
IFNG expression R	GGACATTCAAGTCAGTTACCGA		
ICAM1 expression F	TCTGTTCCCAGGACCTGGCAATG	65	282
ICAM1 expression R	GGAGTCCAGTACACGGTGAGGAAG		
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122
GAPDH expression R	CAAATGAGCCCCAGCCTTCT		

A/T F*= Common forward primer, A R*=reverse primer with A allele T R*= reverse primer with T allele; HGH=Human growth hormone

4.2.6 Estimation of serum IFNγ levels by enzyme-linked immunosorbent assay:

Serum levels of IFNγ in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the Immunotech Human IFNγ ELISA kit (Immunotech SAS, Marseille Cedex 9, France) as per the manufacturer's protocol.

4.2.7 Statistical analysis:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for the polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis. The distribution of the genotypes and allele frequencies of *IFNG* polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.025 were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated.

Age of onset analysis, relative gene expression of *IFNG* and serum IFNγ levels in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al.*, 2007).

4.3 RESULTS

4.3.1 Analysis of association between intron 1 +874 A/T (rs2430561) polymorphism of *IFNG* and susceptibility to vitiligo:

ARMS-PCR for the *IFNG* (+874A/T) polymorphism revealed a 428 bp product for *HGH* as a control gene, and a 265 bp product for *IFNG* T874 (homozygous for allele

T; TT), or A874 (homozygous for allele A; AA), or both alleles T and A (heterozygous; TA). The genotypes for *IFNG* intron1 +874A/T polymorphism were confirmed by running PCR amplicons on 2.0% agarose gel electrophoresis (Figure 1). Intron 1 +874A/T polymorphism of *IFNG* gene was not found to be associated with generalized vitiligo patients (p=0.485) when genotypes were compared with chi-squared test-3x2 contingency table (Table 3). Also, there was no significant difference in allele frequencies of this polymorphism between patients and controls when compared with 2x2 contingency table (p=0.274) (Table 3). Both patients and control population were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.942 and p=0.847 respectively) (Table 3). Interestingly, the frequency of minor 'T' allele was significantly higher in active cases as compared to stable cases of vitiligo (45% vs 31%; p=0.0003) (Table 4). In addition, genotype frequencies and allele frequencies for this polymorphism were also analyzed between male and female patients with vitiligo and controls which revealed no significant difference as detected by chi-squared test (p=0.459 and p=0.030 respectively) (Table 5). This study has 91.22% statistical power for the effect size 0.08 to detect association of *IFNG* +874A/T polymorphism at p<0.05 in patients and control population.

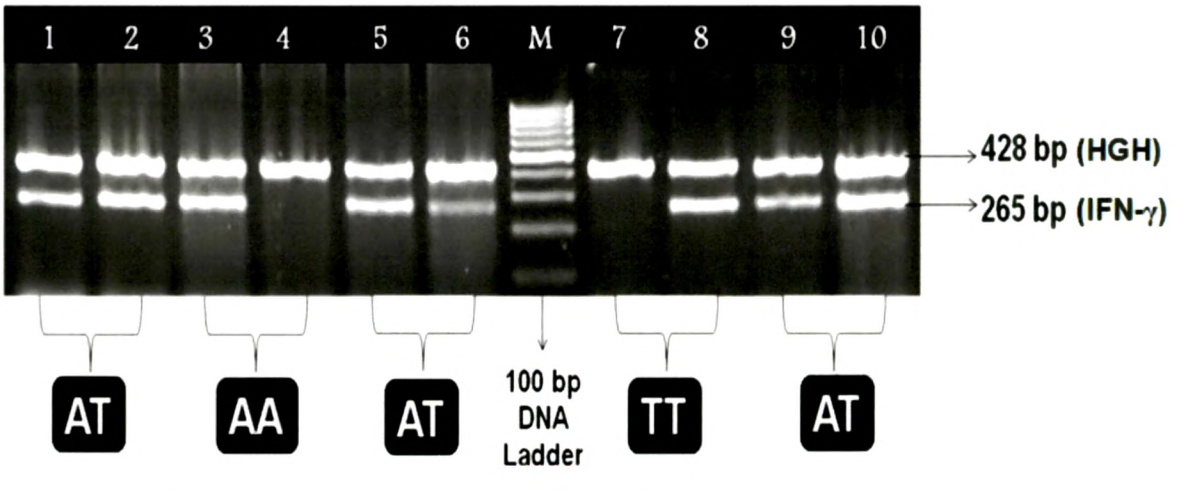


Figure 1. Representative gel picture for genotyping of *IFNG* +874A/T polymorphism by ARMS-PCR on 2.0% agarose gel electrophoresis: lane M: 100 bp DNA Ladder; lanes 3 and 4: AA (homozygous-low producer) genotype; lanes 1, 2, 5, 6, 9 and 10: AT (heterozygous-medium producers) genotypes; lanes 7 and 8: TT (homozygous-high producer) genotype.

Table 3. Distribution of genotypes and allele frequencies for *IFNG* +874 A/T (rs2430561) intron 1 polymorphism in generalized vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 517)	(n = 881)		0.607	
	AA	181 (0.35)	281 (0.32)		(P)	
rs2430561	AT	245 (0.47)	435 (0.49)	0.485[a]	0.884	
(A/T)	TT	91 (0.18)	165 (0.19)		(C)	
	Allele					0.9168
	A	607 (0.59)	997 (0.57)	0.274[b]		(0.7847-
	T	427 (0.41)	765 (0.43)			1.071)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 4. Distribution of genotypes and allele frequencies for *IFNG* +874 A/T (rs2430561) intron 1 polymorphism in active and stable vitiligo patients.

SNP	Genotype or allele	Active Vitiligo Patients (Freq.)	Stable Vitiligo Patients (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 382)	(n = 135)			
	AA	115 (0.30)	66 (0.49)		0.837	
rs2430561	AT	191 (0.50)	54 (0.40)	0.0003[a]	(AV)	
(A/T)	TT	76 (0.20)	15 (0.11)			
	Allele				0.438	0.5543
	A	421 (0.55)	186 (0.69)	<0.0001[b]	(SV)	(0.4129-
	T	343 (0.45)	84 (0.31)			0.7442)

'n' represents number of Patients,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(AV) refers to Active vitiligo Patients and (SV) refers to Stable vitiligo Patients,

[a] Active Patients vs. Stable Patients using chi-squared test with 3×2 contingency table,

[b]Active Patients vs. Stable Patients using chi-squared test with 2×2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 5. Association studies for *IFNG* +874 A/T (rs2430561) intron 1 polymorphism in male and female patients with vitiligo.

SNP	Genotype or allele	Male Patients (Freq.)	Female Patients (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 222)	(n = 295)			
	AA	72 (0.32)	109 (0.37)	0.459[a]	0.620	
rs2430561	AT	112 (0.50)	133 (0.45)		(M)	
(A/T)	TT	38 (0.18)	53 (0.18)			
	Allele				0.267	1.362 (1.041-
	A	256 (0.58)	351 (0.59)	0.030[b]	(F)	1.781)
	T	128 (0.42)	239 (0.41)			

'n' represents number of Patients,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(M) refers to Male Patients and (F) refers to Female Patients,

[a] Male Patients vs. Female Patients using chi-squared test with 3×2 contingency table,

[b]Male Patients vs. Female Patients using chi-squared test with 2×2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

4.3.2 Analysis of association between intron 1 CA microsatellite (rs3138557) polymorphism of *IFNG* and susceptibility to vitiligo:

High Resolution Melt (HRM) curve analysis for CA microsatellite (rs3138557) polymorphism was performed using LightCycler® 480Real-Time PCR. The five genotypes identified as: 12-12 CA, 13-13 CA, 12-13 CA, 13-14 CA and 14-15 CA, based on normalized and temperature shifted difference plots followed by DNA sequencing of samples from each representative groups (Figure 2A). DNA sequencing was carried out for samples from each representative groups obtained by HRM analysis which revealed five different CA repeats: 12-12 CA, 13-14 CA, 12-13 CA, 13-13 CA and 12-14 CA repeats (Figure 2B).

The distribution of the *IFNG* microsatellite polymorphism (CA) was different between cases and controls (p<0.001). The genotype (CA) 12-12, i.e. the homozygous state for allele 2 was significantly more frequent (p=0.005) in vitiligo patients (57.0%)

than in controls (23.0%) whereas the genotype (CA) 12-13 recurred less frequently in the patients (10.0%) versus controls (42%; p=0.005) (Table 6). The allelic frequency differed significantly between cases and controls (p=0.004). In vitiligo patients the allele 12 was significantly (p=0.004) more frequent (66.0%) than in controls (48.0%), whereas the allele 13 recurred less frequently (28.0%) than in controls (46.0%; p=0.005) (Table 6). The results suggest significant association of *IFNG* 12-12 CA repeats with vitiligo susceptibility.

Table 6. Distribution of genotypes and alleles frequencies for *IFNG* CA microsatellite (rs3138557) polymorphism in generalized vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* Value*	*p* Value#
	Genotype	(n = 160)	(n = 210)		
	CA 12-12	92 (0.57)	48 (0.23)	<0.001	0.005
rs3138557	CA 13-13	32 (0.20)	49 (0.23)	<0.001	NS
(CA	CA 12-13	16 (0.10)	88 (0.42)	<0.001	0.005
Repeats)	CA 12-14	11 (0.07)	19 (0.09)	<0.001	NS
	CA 13-14	09 (0.06)	06 (0.03)	<0.001	NS
	Allele				
	CA 12	211 (0.66)	203 (0.48)	0.004	0.004
	CA 13	89 (0.28)	192 (0.46)	0.004	0.005
	CA 14	20 (0.06)	25 (0.06)	0.004	NS

*Comparison of the distribution of genotypes between the two groups.

#Comparison of the single genotype/allele against all the others.

NS= not significant.

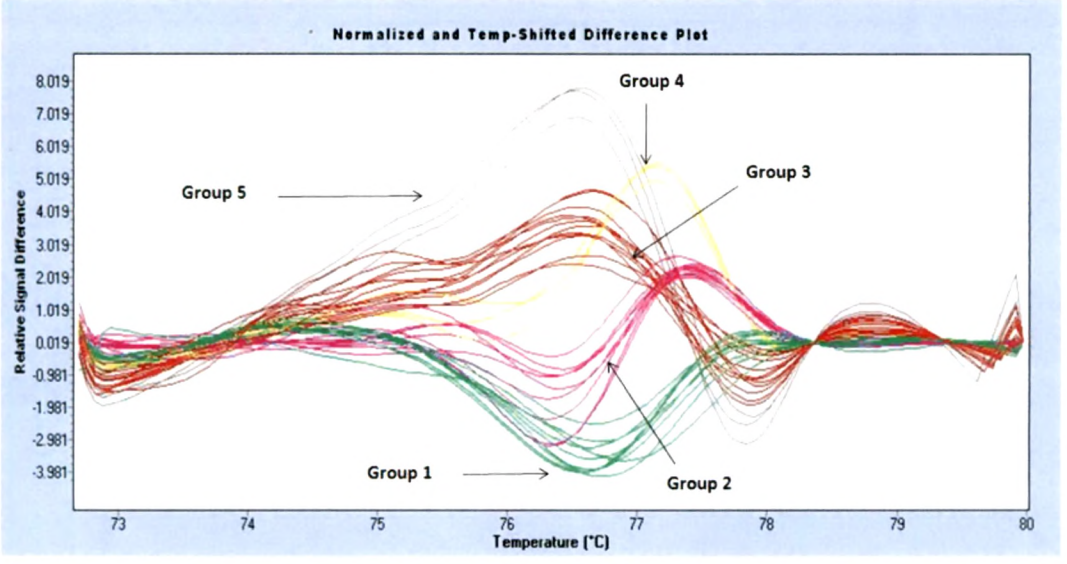
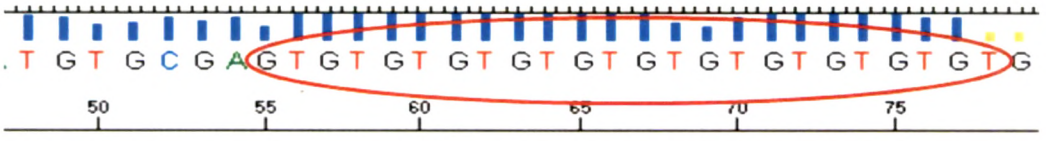
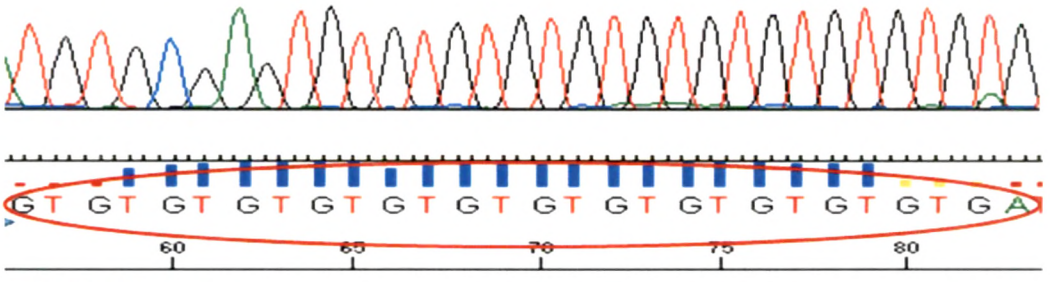


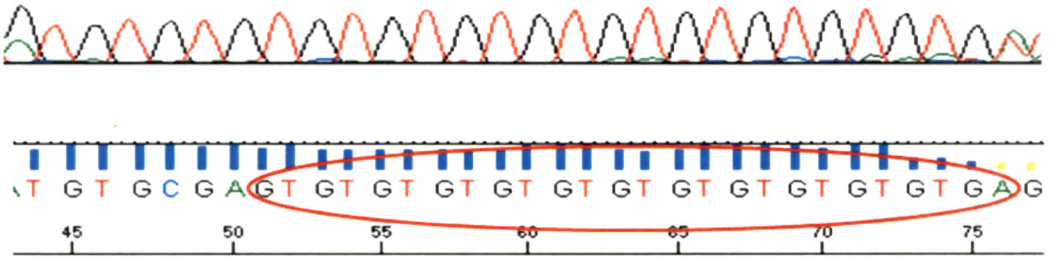
Figure 2. (A) High Resolution Melt (HRM) curve analysis for *IFNG* CA microsatellite (rs3138557) polymorphism using LightCycler®480 Real-Time PCR protocol. The five genotypes identified as: 12-12 CA, 13-13 CA, 12-13 CA, 13-14 CA and 12-14 CA, based on normalized and temperature shifted difference plots followed by DNA sequencing of samples from each representative groups.



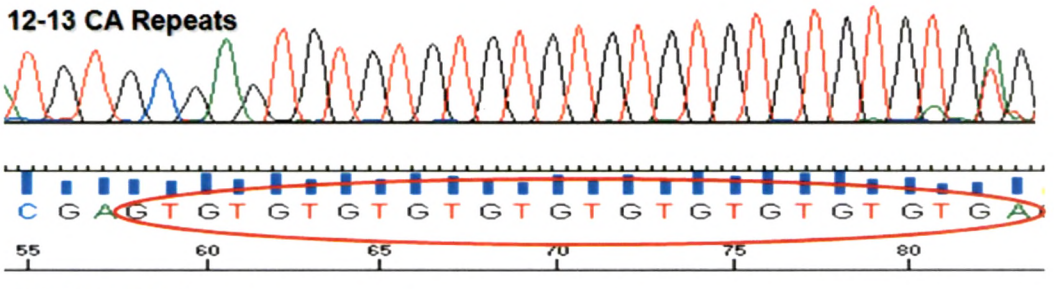
12 CA Repeats



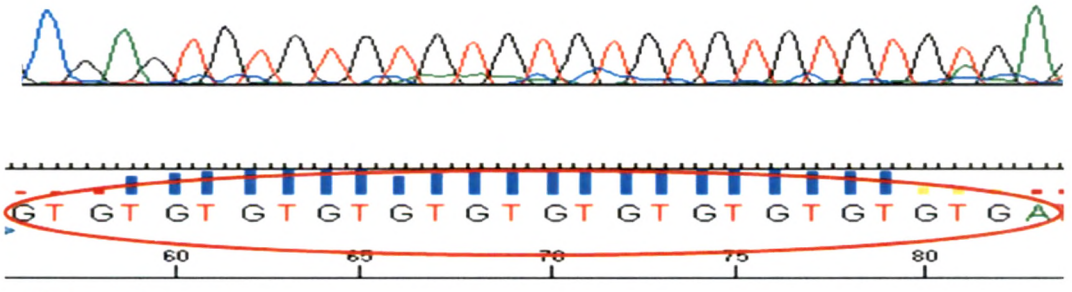
13-14 CA Repeats



12-13 CA Repeats



13 CA Repeats



12-14 CA Repeats

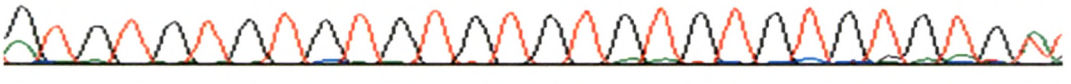


Figure 2. **(B)** DNA sequencing of samples from each representative groups obtained by HRM analysis. Different *IFNG* CA repeats obtained are shown in electrophoretograms as: 12-12 CA, 13-14 CA, 12-13 CA, 13-13 CA and 12-14 CA repeats.

4.3.3 Effect of *IFNG* +874 A/T polymorphisms on age of onset of vitiligo:

When age of onset of the disease was correlated with the *IFNG* +874A/T genotypes, patients with higher (IFN-γ) producer TT genotypes showed an early onset of the disease as compared to AA and AT genotypes ($p<0.0001$ and $p=0.004$ respectively) (Figure 3A). Moreover, patients with genotype AT showed an early onset of the disease as compared to AA genotypes ($p=0.011$) (Figure 3A) suggesting the effect of the susceptible allele 'T' on the early onset of disease.

Interestingly, when male and female vitiligo patients were analyzed for age of onset of the disease, female patients had significant early onset of the disease as compared to the male patients ($p=0.002$) (Figure 3B).

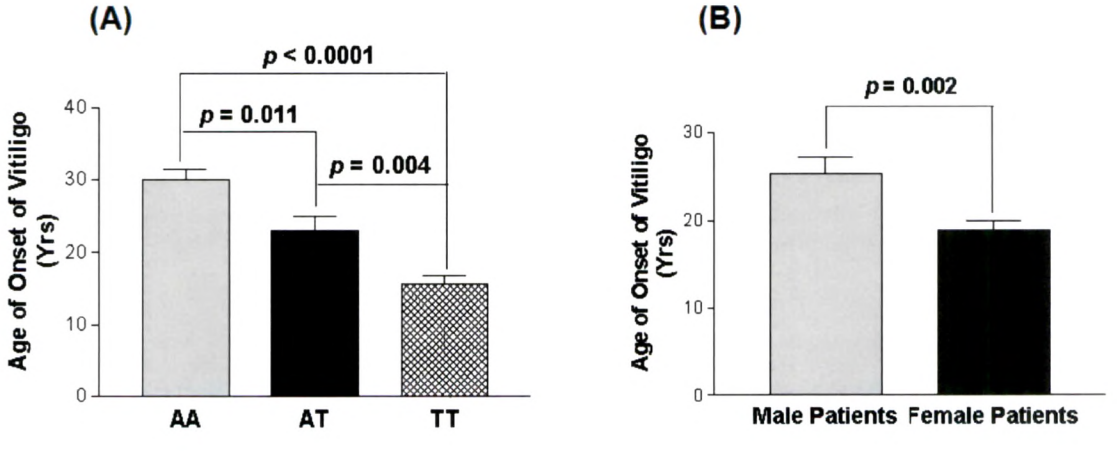


Figure 3. **Age of onset of the disease in vitiligo patients:**

(A) Comparison of age of onset of the disease (yrs) with respect to *IFNG* A/T (rs2430561) polymorphism in 517 vitiligo patients. Vitiligo patients with TT

genotype showed early age of onset of disease as compared to AA (Mean age of onset ± SEM: 15.66 ± 1.145 vs 29.94 ± 1.592; $p<0.0001$) and AT genotypes (Mean age of onset ± SEM: 15.66 ± 1.145 vs 23.04 ± 2.013; $p=0.004$). Patients with AT genotype showed early age of onset of disease as compared to AA genotype (Mean age of onset ± SEM: 23.04 ± 2.013 vs 29.94 ± 1.592; $p=0.011$).

(B) Comparison of age of onset of the disease (yrs) with respect to gender differences in 222 male patients and 295 female patients with vitiligo. Female patients showed early age of onset of disease as compared to male patients (Mean age of onset ± SEM: 18.87 ± 1.128 vs 25.32 ± 1.888; $p=0.002$).

4.3.5 Relative gene expression of *IFNG* in patients with vitiligo and controls:

Comparison of the findings showed significant increase in expression of *IFNG* transcripts in 122 vitiligo patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values ($p<0.0001$) (Figure 4A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.047 fold change in the expression of *IFNG* transcript in patients as compared to controls (Figure 4B).

4.3.6 Correlation of *IFNG* transcripts with +874 A/T polymorphism:

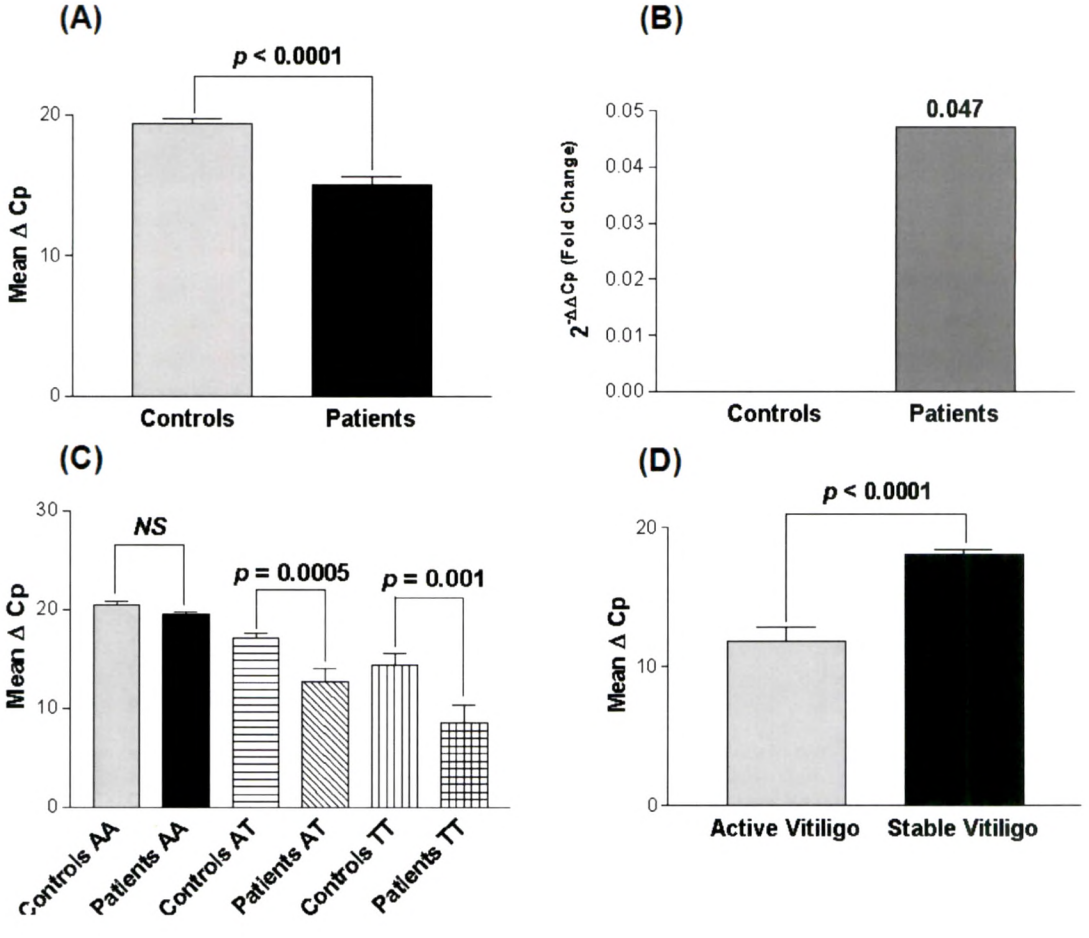
Further, the expression levels of *IFNG* were analyzed with respect to +874 A/T (rs2430561) genotypes (Figure 4C). Interestingly, *IFNG* expression was significantly increased in patients with susceptible TT genotypes as compared to controls ($p=0.001$). Also, patients with genotypes AT showed increased *IFNG* transcripts as compared to controls ($p=0.0005$); however, no significant difference was observed in *IFNG* expression in patients as compared to controls with AA genotypes ($p=0.057$).

5.3.6 Effect of *IFNG* expression on disease progression:

In addition, we also checked the effect of *IFNG* expression on progression of the disease i.e. active and stable cases (Figure 4D). Interestingly, active vitiligo patients showed significant increase in expression of *IFNG* transcripts as compared to the patients with stable vitiligo ($p<0.0001$) suggesting the involvement of IFNγ in disease

progression. Moreover, the susceptibility of the disease was also checked based on the gender differences and we found that female patients with vitiligo showed significantly higher *IFNG* expression as compared to male patients (p=0.0005) (Figure 4E).

When *IFNG* expression was monitored in different age of onset groups, patients with the age group 1-20 yrs showed significantly increased expression of *IFNG* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.0002, p<0.0001 and p<0.0001 respectively) suggesting the importance of IFNγ in early onset of the disease (Figure 4F).



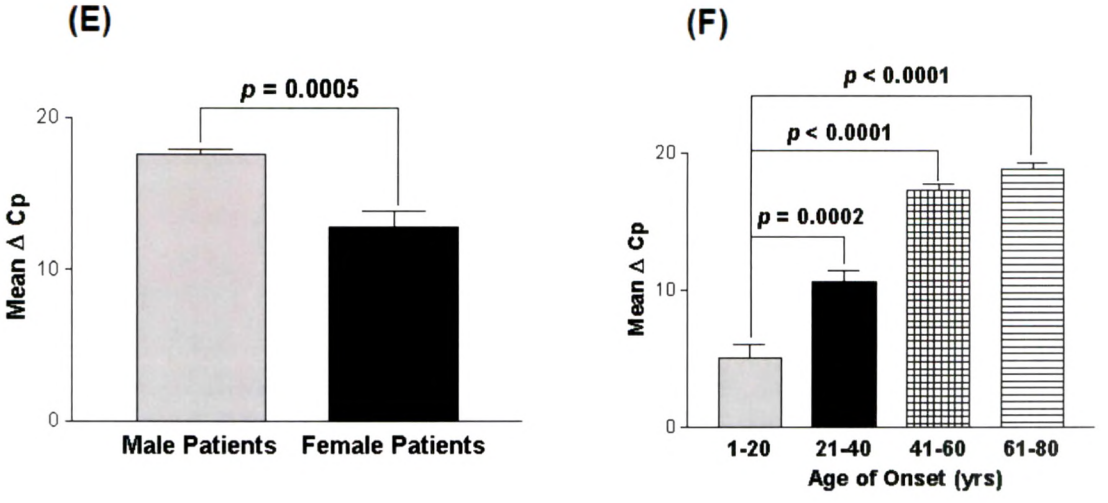


Figure 4. Relative gene expression of *IFNG* in controls and vitiligo patients:

(A) Expression of *IFNG* transcripts in 175 controls and 122 generalized vitiligo patients as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *IFNG* as compared to controls (Mean ΔCp ± SEM: 15.04 ± 0.6603 vs 19.44 ± 0.2806; $p<0.0001$).

(B) Expression fold change of *IFNG* transcripts in 166 vitiligo patients against 175 controls showed 0.047 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *IFNG* transcripts with respect to *IFNG* +874A/T (rs2430561) polymorphism in 122 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *IFNG* with TT (Mean ΔCp ± SEM: 8.531 ± 1.778 vs 14.40 ± 1.242; $p=0.001$) and AT (Mean ΔCp ± SEM: 12.72 ± 1.317 vs 17.10 ± 0.5038; $p=0.0005$) genotypes as compared to controls. There was no significant difference in the expression of *IFNG* in patients with AA genotypes (Mean ΔCp ± SEM: 19.50 ± 0.2929 vs 20.45 ± 0.3717; $p=0.057$) as compared to controls.

(D) Expression of *IFNG* transcripts with respect to progression of the disease in 91 patients with active vitiligo and 31 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients showed significantly increased mRNA levels of *IFNG* as compared to stable vitiligo patients (Mean ΔCp ± SEM: 11.79 ± 1.034 vs 18.06 ± 0.3447; $p<0.0001$).

(E) Expression of *IFNG* transcripts with respect to gender differences in 52 male patients and 70 female patients with vitiligo, as suggested by Mean ΔCp. Female patients with vitiligo showed significantly increased mRNA levels of *IFNG* as

compared to male vitiligo patients (Mean ΔCp ± SEM: 12.83 ± 1.008 vs 17.63 ± 0.3478; p=0.0005).

(F) Expression of *IFNG* transcripts with respect to different age groups in 122 vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients with the age of onset 1-20 yrs showed significantly increased expression of *IFNG* mRNA as compared to the age groups 21-40 yrs (Mean ΔCp ± SEM: 5.047 ± 0.9394 vs 10.65 ± 0.8343; p=0.0002), 41-60 (Mean ΔCp ± SEM: 5.047 ± 0.9394 vs 17.27 ± 0.5239; p<0.0001) and 61-80 yrs (Mean ΔCp ± SEM: 5.047 ± 0.9394 vs 18.87 ± 0.4213; p<0.0001).

4.3.7 Functional correlation of *IFNG* +874 A/T polymorphism with its levels in the serum:

To find any functional correlation of the investigated *IFNG* promoter polymorphisms with its level in the serum, IFNγ levels were measured in 214 vitiligo patients and 236 unaffected controls. Vitiligo patients showed significant increased serum IFNγ levels as compared to controls (p=0.002) (Figure 5A). Moreover, when the patient groups were analyzed based on disease progression with respect to IFNγ levels, patients with active vitiligo had significantly higher IFNγ levels as compared to stable vitiligo (p=0.003) (Figure 5B).

Furthermore, when +874 A/T genotypes were analyzed with IFNγ levels, patients with TT and AT genotypes showed significantly higher levels of IFNγ as compared to those of controls (p=0.001; p=0.023) (Figure 5C). However, patients with AA genotype showed no significant difference in IFNγ levels as compared to those of controls (p=0.521). Additionally, when the male and female patients were analyzed with respect to IFNγ levels, female patients showed significantly higher levels of IFNγ as compared to male patients (p=0.0005) (Figure 5D).

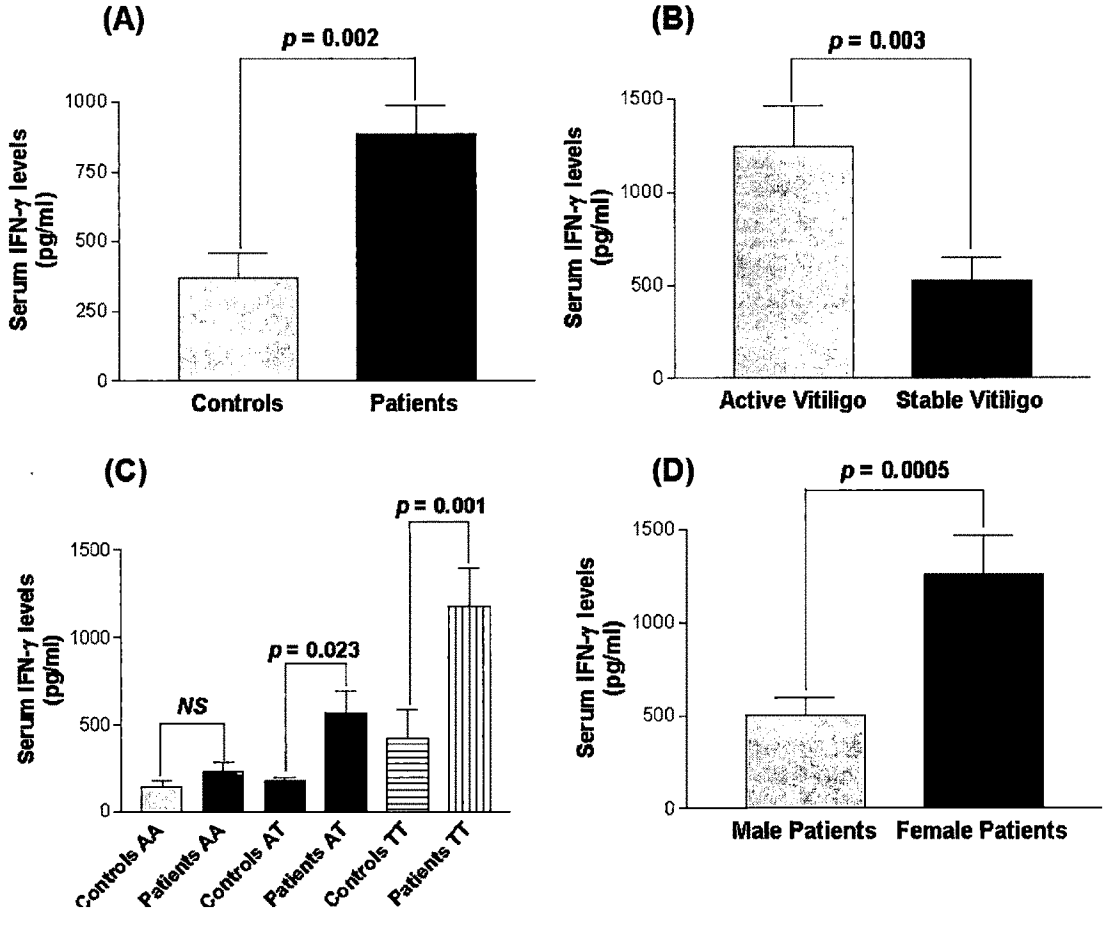


Figure 5. Serum IFNγ levels in controls and vitiligo patients:

(A) Serum IFNγ levels in 236 controls and 214 generalized vitiligo patients. Vitiligo patients showed significantly increased levels of IFNγ as compared to controls (Mean ± SEM: 885.8 ± 103.7 vs 367.1 ± 90.53; p=0.002).

(B) Serum IFNγ levels with respect to progression of the disease in 156 patients with active vitiligo and 58 patients with stable vitiligo. Active vitiligo patients showed significantly increased levels of IFNγ as compared to stable vitiligo patients (Mean ± SEM: 1245 ± 216.8 vs 523.1 ± 121.4; p=0.003).

(C) Serum IFNγ levels with respect to *IFNG* +874A/T (rs2430561) polymorphism in 214 vitiligo patients and 236 controls. Vitiligo patients showed significantly increased levels of IFNγ with TT (Mean ± SEM: 1174 ± 217.4 vs 421.4 ± 161.1; p=0.001) and AT (Mean ± SEM: 566.0 ± 124.1 vs 175.2 ± 21.85; p=0.023) genotypes as compared to controls. No significant difference in IFNγ levels was observed in patients with AA genotypes (Mean ± SEM: 229.4 ± 48.73 vs 144.6 ± 30.41; p=0.521) as compared to controls.

4.3.9 Relative gene expression of *ICAM1* in patients with vitiligo and controls:

Comparison of the findings showed significant increase in expression of *ICAM1* transcripts in 122 vitiligo patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.008) (Figure 7A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.369 fold change in the expression of *ICAM1* transcript in patients as compared to controls (Figure 7B).

In addition, the effect of *ICAM1* expression on progression of the disease i.e. active and stable cases (Figure 7C) revealed that active vitiligo patients had significantly increased expression of *ICAM1* transcripts as compared to patients with stable vitiligo (p=0.008) suggesting the involvement *ICAM1* in disease progression. Moreover, the susceptibility of the disease was also checked based on the gender differences and we found that female patients with vitiligo showed significantly higher *ICAM1* expression as compared to male patients (p=0.006) (Figure 7D). When *ICAM1* expression was monitored in different age of onset groups of patients, patients with the age group 1-20 yrs showed significantly increased expression of *ICAM1* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.0002, p<0.0001 and p<0.0001 respectively) suggesting the importance of *ICAM1* in early onset of the disease (Figure 7E).

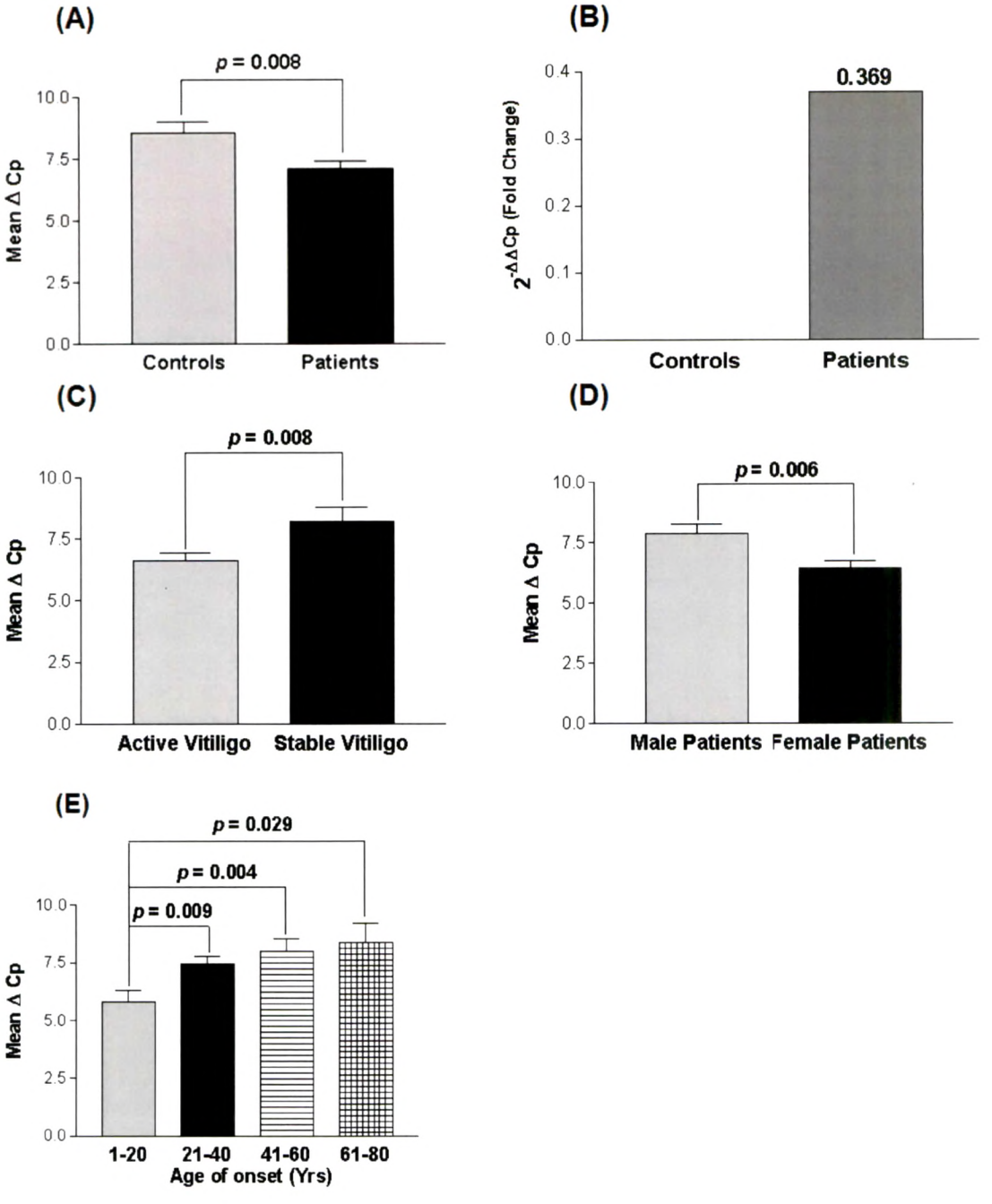


Figure 7. Relative gene expression of *ICAM1* in controls and vitiligo patients:

(A) Expression of *ICAM1* transcripts in 175 controls and 122 generalized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *ICAM-1* as compared to controls (Mean ΔCp ± SEM: 8.552 ± 0.4568 vs 7.112 ± 0.2705; p=0.008).

(B) Expression fold change of *ICAM1* transcripts in 122 vitiligo patients against 175 controls showed 0.369 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *ICAM1* transcripts with respect to activity of the disease in 91 patients with active vitiligo and 31 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients showed significantly increased mRNA levels of *ICAM1* as compared to stable vitiligo patients (Mean ΔCp ± SEM: 6.662 ± 0.2852 vs 8.227 ± 0.5610; p=0.008).

(D) Expression of *ICAM1* transcripts with respect to gender differences in 52 male patients and 70 female patients with vitiligo, as suggested by Mean ΔCp. Female patients with vitiligo showed significantly increased mRNA levels of *ICAM1* as compared to male vitiligo patients (Mean ΔCp ± SEM: 7.890 ± 0.4093 vs 6.435 ± 0.3266; p=0.006).

(E) Expression of *ICAM1* transcripts with respect to different age groups in 122 vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients with the age of onset 1-20 yrs showed significantly increased expression of *ICAM1* mRNA as compared to the age groups 21-40 yrs (Mean ΔCp ± SEM: 5.819 ± 0.4737 vs 7.436 ± 0.3662; p=0.009), 41-60 (Mean ΔCp ± SEM: 5.819 ± 0.4737 vs 7.985 ± 0.5435; p=0.004) and 61-80 yrs (Mean ΔCp ± SEM: 5.819 ± 0.4737 vs 8.384 ± 0.8328; p=0.029).

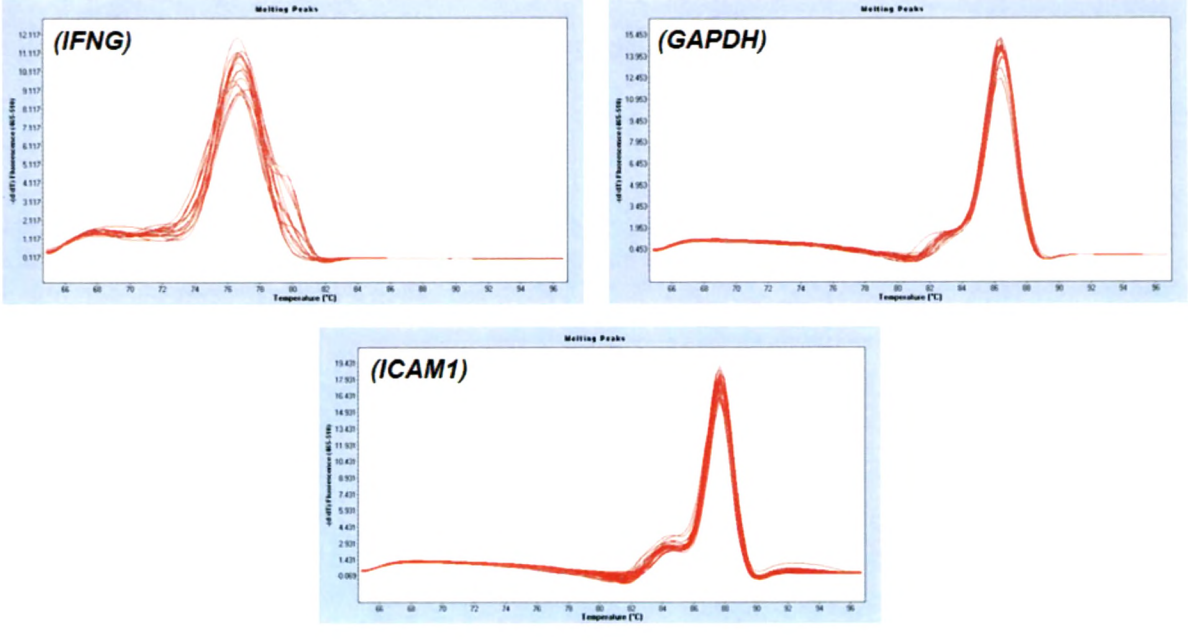


Figure 8. Melt curve analysis of *IFNG*, ICAM1 and *GAPDH* showing specific amplification.

4.4 DISCUSSION

Vitiligo cannot be explained by simple Mendelian genetics, however it is characterized by incomplete penetrance, multiple susceptibility loci and genetic heterogeneity (Zhang *et al.*, 2005). Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo since it shows frequent association with other autoimmune disorders such as rheumatoid arthritis, Graves' disease etc. (Ochi and Groot, 1969). About 25-30% of generalized vitiligo patients manifest at least one other autoimmune disease; particularly Graves' disease, autoimmune hypothyroidism, rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, Addison's disease, and systemic lupus erythematosus (Alkhateeb *et al.*, 2003; Laberge *et al.*, 2005). Previously, Shajil *et al.* (2006) suggested that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil *et al.*, 2006). The present study also shows that 12.57% of generalized vitiligo patients have one or more first degree relative affected suggesting the involvement of genetic factors in pathogenesis of generalized vitiligo.

The autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity (Shajil *et al.*, 2006; Kemp *et al.*, 2001). We also detected circulating autoantibodies in the sera of 75% of Gujarat vitiligo patients as compared to unaffected individuals. Interestingly, increased autoantibody titers against melanocytic antigens have been reported (Harning *et al.*, 1991) and also elevated serum levels of soluble interleukin (sIL)-2 receptor were found in correlation with disease activity (Honda *et al.*, 1997). Increased sIL-2R is an indication of T cell activation (Honda *et al.*, 1997) and it has been reported that activated T cells are present in vitiligo patients (Le Poole *et al.*, 1993). Moreover, *in vitro* direct analysis of margins of vitiliginous skin showed the presence of polarized type-1 T cells ($CD4^+$ and particularly $CD8^+$), which predominantly secrete interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) which might be associated with the destruction of melanocytes during active disease (Wajkowicz-Kalijska *et al.*, 2003). Thus, cytokines are important mediators of immunity, and there is now convincing evidence that cytokines are involved in the pathogenesis of autoimmune diseases (Feldmann *et al.*, 1998). The cytokine mRNA and protein levels depend on both genetic and environmental factors (Gottenberg *et al.*, 2004). Analysis of cytokine gene

polymorphisms would be able to detect a genetic abnormality of cytokine regulation that might play a role in the pathophysiology of the disease. Therefore, cytokines encoding genes could be considered as the candidate genes for vitiligo susceptibility. The ultimate pathway of destruction of melanocytes in vitiligo is not known. Apoptosis is one of the cell death pathways suggested for melanocyte destruction. Cytokines such as IFNγ, TNFα, or IL1 released by lymphocytes and keratinocytes can initiate apoptosis (Huang *et al.*, 2002). An imbalance of cytokines in the epidermal microenvironment of lesional vitiligo skin has been demonstrated, which could impair the life span and function of melanocytes (Grimes *et al.*, 2004; Moretti *et al.*, 2002). In vitiligo skin, a significantly higher expression of *IFNG* was detected, compared to healthy controls indicating that cytokine imbalance is involved in the depigmentation process observed in vitiligo (Grimes *et al.*, 2004). We therefore, selected *IFNG* as a candidate gene to study vitiligo susceptibility in Gujarat population.

Pravica *et al.*, (1999) reported that the single nucleotide polymorphism A/T at the 5' end of the CA repeat of the human *IFNG* gene (+874A/T) directly affects the level of IFNγ production and correlates with the presence of the A874 allele and low production of IFNγ as compared to T874 allele. The authors proposed that this polymorphism (T allele) coincided with a putative nuclear factor-κB (NF-κB) binding site that could have functional consequences for transcription of the human *IFNG* gene, which could directly increase the level of IFNγ production (Pravica *et al.*, 2000). There was a significant association between genotype and the frequency of the A allele of the +874A/T polymorphism in atopic patients in Egyptian population (Hussein *et al.*, 2009). The study also confirms decreased serum IFNγ levels along with the presence of homozygous AA compared with homozygous TT genotypes. Furthermore, the T allele of +874A/T polymorphism was found to be associated with elevated *IFNG* expression with the A allele, in Korean patients with SLE (Kim *et al.*, 2010).

However, Tamandani *et al.* (2008) reported significant association of *IFNG* +874A/T polymorphism with increased risk of cervical cancer in North Indian population. Gangwar *et al.* (2009) proposed that the *IFNG* +874A/T SNP may represent an important susceptibility biomarker for cervical cancer risk as well as other diseases in North Indian population and should be explored further. However, another study

(Vidyarani *et al.*, 2006) on South Indian population suggested a lack of functional association of *IFNG* +874A polymorphism in tuberculosis. These Indian cohort studies regarding *IFNG* +874A/T SNP prompted us to investigate the role of *IFNG* gene in Gujarat population with generalized vitiligo.

The particular *IFNG* +874A/T SNP has been studied extensively in autoimmune diseases also. Rekha *et al.* (2006) suggested a differential association of high (T) and low (A) producing alleles of *IFNG* gene with Hashimoto's thyroiditis and Graves' disease. The high *IFNG* producing allele T was associated with Hashimoto's thyroiditis where as in Graves' hyperthyroidism the association was stronger with the low producing allele A. IFNγ levels have also been monitored in certain autoimmune diseases, out of which active cases of systemic lupus erythematosus and mixed connective tissue disease (MCTD) showed increased levels of IFNγ suggesting its role in the autoimmune disease pathogenesis (Funauchi *et al.*, 1991). In addition, a family based study (Schena *et al.*, 2006) with IgA nephropathy showed a strong association between the 13-CA repeat allele and the A variant of the +874A/T single nucleotide polymorphism. The report also confirms a correlation between the +874A allele and lower production of IFNγ as compared to +874T allele (Schena *et al.*, 2006).

Namian *et al.* (2009) showed that +874A/T intron 1 polymorphism of *IFNG* gene is not associated with susceptibility to vitiligo in Iranian patients. This was the first genetic association study of *IFNG* gene polymorphism with vitiligo. Hence, our present study explores genetic association of *IFNG* gene polymorphisms with vitiligo and our results are in accordance with those of Namian *et al.* (2009).

Our results show that +874A/T intron1 polymorphism of *IFNG* gene is not associated with vitiligo susceptibility in Gujarat population. However, the genotype-phenotype correlation for +874A/T polymorphism suggested that *IFNG* mRNA and protein levels were higher in homozygous TT and heterozygous AT genotype patients than that of controls indicating the profound effect of allele 'T' with increased levels of IFN-γ. Moreover, the 'T' allele frequency was significantly higher in patients with active vitiligo as compared to stable vitiligo indicating the role of +874 'T' allele in disease progression. In addition, active cases of vitiligo showed increased mRNA and protein levels of IFNγ as compared to stable cases, further confirming the important

role of IFNγ in disease progression. Age of onset analysis with *IFNG* +874 A/T genotypes revealed that patients with TT and AT genotypes had an early onset of the disease as compared to AA wild type genotype suggesting the involvement of 'T' allele in early phase of the disease. Also, patients with an early age of onset of the disease (1-20 yrs) had significantly higher levels of *IFNG* transcript as compared to those of late onset groups suggesting the crucial role of IFNγ in early phase of the disease.

In addition, gender based study showed that female patients had an early onset of the disease as compared to male patients suggesting that females may be more inclined towards vitiligo. Furthermore, gender based expression study indicated that females had higher levels of *IFNG* mRNA and protein levels as compared to males suggestive of the fact that females are more prone to autoimmunity.

IFNγ enhances human B cell proliferation (Francois *et al.*, 1988) and IFNγ therapy may cause vitiligo (SeÈkin *et al.*, 2004; Passeron and Ortonne, 2005; Kocer *et al.*, 2009). IFNγ is predominantly produced by natural killer cells, $CD4^+$ and $CD8^+$ T cells. Its mRNA expression has increased in vitiligo involved and uninvolved skin compared with control skin (Grimes *et al.*, 2004; Schoenborn *et al.*, 2007). IFNγ stimulates the expression of intercellular adhesion molecule 1 (ICAM1), which is important for activating T cells and recruiting leukocytes (Hedley *et al.*, 1998; Ahn *et al.*, 1994). ICAM1 protein levels are upregulated in vitiligo skin and in melanocytes from perilesional vitiligo skin (Al Badri, 1993). The present study also showed increased expression of ICAM1 in vitiligo patients suggesting that increased IFNγ levels might be responsible for increased *ICAM1* expression in vitiligo patients. It has been reoprted that increased expression of this adhesion molecule on the melanocytes enhances T cell -melanocyte attachment in the skin and may lead to the destruction of melanocytes in vitiligo (Al Badri, 1993). Moreover, the *ICAM1* expression was increased in active cases of vitiligo as compared to stable vitiligo suggesting its role in progression of the disease. The *ICAM1* expression was increased with early age of onset of the disease further implicating the important role of ICAM1 in early phase of the disease. Also, female patients showed an increased expression of ICAM1 as compared to male patients suggesting that females have more susceptibility towards vitiligo.

Among the dinucleotide repeats, $(TG/CA)_n$ (CA-) repeats represent the most common class of microsatellites in vertebrates, including humans (Toth *et al.*, 2000). Because of the strucure of alternating purine/pyrimidine sequences, CA repeats have a tendency to form Z-form under physiological conditions, suggesting a possible role for such Z-DNA elements in chromatin activation or genome rearrangements that could influence gene expression (Nordheim and Rich, 1983). Biochemically, this feature of the CA-repeats may affect the movement of RNA polymerases, thus modulating gene expression levels (Peck and Wang, 1985). It also has been shown that intronic CA sequences constitute novel and widespread regulatory elements of alternative splicing (Hui *et al.*, 2005).

The *IFNG* has a $(CA)_n$ repeat element within the first intron (Ruiz-Linares *et al.*, 1993). The location of the CA repeat in the first intron is consistent with an effect on either gene transcription or on pre-mRNA processing (Agrawal *et al.*, 2000). It has been reported that good IFNγ producers are considered to be individuals with 12 CA repeats and a 'T' at the polymorphic site (Pravica *et al.*, 1999; Pravica *et al.*, 2000). Individuals with the presence of 'A' allele with 13 repeats have low IFNγ generation potential (Miyake *et al.*, 2002; Lee *at al.*, 2001). The current study finds significant association of 12 CA repeats with vitiligo susceptibility suggesting the increased IFNγ levels in patients. Interestingly, the serum IFNγ were found to be increased with individuals harboring 12 CA repeats as compared to those of longer repeats suggesting the important role of 12 CA repeats in increased IFNγ production. There are no reports available for *IFNG* CA microsatellite polymorphism for vitiligo susceptibility. However, the CA microsatellite polymorphism has been associated with increased susceptibiltiy to several autoimmune diseases such as athama, type-1 diabetes, polymyalgia rheumatica (PMR) etc. (Nagarkatti *et al.*, 2002; Tegoshi *et al.*, 2002; Gonzalez-Gay *et al.*, 2004).

As IFNγ has a role in inducing melanocyte apoptosis and destruction in vitiligo, genetic association studies with *IFNG* polymorphisms in different ethnic populations need to be explored. Further analysis of other cytokine polymorphisms with adequate sample size may result in a better understanding of the genes involved in vitiligo pathogenesis.

The present study showed significant increase in *IFNG* mRNA and protein levels in vitiligo patients as compared to controls. Recently, Reimann *et al.* (2012) showed that *IFNG* mRNA expression level rose significantly in patient's involved skin and also in PBMCs compared with controls. In addition, Shi and Erf, (2012) also showed increased *IFNG* expression in vitiligo lesions of Smyth line chickens. These reports along with the present study emphasize the role of IFNγ in the pathogenesis of generalized vitiligo.

In conclusion, we propose that *IFNG* CA microsatellite but not +874 A/T may be a genetic risk factor for generalized vitiligo in Gujarat population; however, +874T allele may play a role in increased expression of *IFNG* mRNA and protein levels which can affect the onset and progression of the disease. Moreover, the increased IFNγ levels in patients may lead to increased *ICAM1* expression which is probably an important link between cytokines and T cells involved in vitiligo pathogenesis.

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CHAPTER V

CYTOTOXIC T-LYMPHOCYTE ASSOCIATED ANTIGEN-4 (*CTLA*-4) GENE POLYMORPHISMS AND THEIR GENOTYPE-PHENOTYPE CORRELATION WITH GENERALIZED VITILIGO SUSCEPTIBILITY

5.1 INTRODUCTION

Vitiligo is an acquired, hypomelanotic disease characterized by circumscribed depigmented macules. The absence of melanocytes from the lesional skin due to their destruction has been suggested to be the key event in the pathogenesis of vitiligo (Ortonne and Bose, 1993). In India, the incidence of vitiligo is found to be 0.5% (Das *et al.,* 1985). The autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity (Shajil *et al.,* 2006a; Kemp *et al.,* 2001). The autoimmune hypothesis gains further support from immunotherapy studies of melanoma patients (Rosenberg, 1997). Vitiligo is a polygenic disease and several candidate genes including the major histocompatibility complex (*MHC)*, angiotensin converting enzyme (*ACE)*, catalase (*CAT)*, cytotoxic T-lymphocyte associated antigen-4 (*CTLA4*), catechol o-methyl transferase (*COMT)*, estrogen receptor (*ESR)*, mannose-binding lectin 2 (*MBL2*) and protein tyrosine phosphatase non-receptor type-22 (*PTPN22*) that play a role in regulating immunity have been tested for genetic association with generalized vitiligo (Spritz, 2007; Spritz, 2008).

Recent studies postulate that the peripheral mechanisms of T cell tolerance are essential to control self-reactive T cells and the elimination of self-tolerance may result in the development of autoimmune diseases (Bluestone, 1997). Hence, molecules affecting these mechanisms are obvious candidates for conferring risk to autoimmunity.

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4; CD152) is expressed exclusively on activated CD4+ and CD8+ T cells and binds the same ligands, B7-1 and B7-2, as CD28 but with a 20 to 50 fold higher affinity (Walunas *et al.,* 1994). However, CD28 provides a critical costimulatory signal essential for the initiation and progression of T cell immunity, data indicate that *CTLA*-4 may actually function to down-regulate T cell function (Lenschow *et al.,* 1996; Walunas *et al.,* 1994). The CTLA-4:B7 interaction plays a critical role in regulating self-tolerance, and hence in susceptibility to autoimmune diseases. The human *CTLA4* gene maps to chromosome 2q33 and comprises 3 exons in addition to the leader sequence. The human *CTLA4*

sequence encodes two transcripts of 1.8 and 0.8 kb, respectively known as full length *CTLA4* (*flCTLA4*) and soluble *CTLA4* (*sCTLA4*) (Harper *et al.,* 1991).

The single nucleotide polymorphism: A to G transition at position 49 (A49G) of exon 1 leads to an alanine to threonine amino acid substitution at codon 17 of the leader peptide (A17T) (Nistico *et al.,* 1996). The +49A/G, is in linkage disequilibrium with CT60 in Poles and other Caucasians (Holopainen *et al.,* 2001; Ueda *et al.,* 2003). A few studies have addressed the possible functional significance of these polymorphisms of *CTLA4*. Interestingly, the G allele of *CTLA4* +49A/G SNP was reported to be involved in the altered intracellular transport of the CTLA-4 protein and its availability on the cell surface. In addition, the 3' UTR CT60A/G allelic variation was reported to be correlated with lower mRNA levels of the soluble alternative splice form of *CTLA4* (*sCTLA4*), suggesting its crucial role in autoimmune diseases (Ueda *et al.,* 2003). One report suggested a correlation between the exon 1 +49A/G SNP and *CTLA4* mRNA levels in human peripheral blood mononuclear cells, whereas another study was unable to demonstrate any significant correlation (Barnes *et al.,* 1997; Cavallo *et al.,* 1997). However, both the studies were preliminary and based on relatively few samples, thus further studies addressing this aspect are warranted.

Using the candidate gene approach, the role of *CTLA4* has been investigated in various autoimmune diseases e.g. Graves disease, Hashimoto's thyroiditis (HT), Addison's disease (AD), Insulin-dependent diabetes mellitus (IDDM), Myasthenia gravis (MG), Multiple sclerosis (MS), Systemic lupus erythematosus (SLE), Rheumatoid arthritis (RA) including Vitiligo. As CTLA-4 appears to be a negative regulator of the normal immune response, it is of interest to investigate the genotype-phenotype correlation of *CTLA4* in vitiligo. Hence, the aims of this study were: i.) to measure and compare *CTLA4* mRNA levels in patients with vitiligo and in unaffected controls; ii.) to determine whether the two well-characterized *CTLA4* polymorphisms [exon1 +49AG (rs231775) and 3' UTR CT60A/G (rs3087243)] are associated with vitiligo susceptibility and modulate *CTLA4* mRNA levels in these groups.

5.2 MATERIALS AND METHODS

5.2.1 Study Subjects:

The study group included 437 vitiligo patients comprised of 190 males and 247 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. The study involved generalized vitiligo patients including acrofacial vitiligo and vitiligo universalis. A total of 746 ethnically and sex-matched unaffected individuals were included as controls in this study. The control group comprised 331 males and 415 females (Table 1). None of the healthy individuals had any evidence of vitiligo and any other autoimmune diseases.

The study plan was approved by the ethical committee of Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of generalized vitiligo patients and unaffected controls.

	Generalized Vitiligo Patients	**Controls**
	(n =437)	(n =746)
Average age (mean age ± SD)	32.45 ± 13.48 yrs	28.23 ± 14.42yrs
Sex: male	190 (43.48%)	331 (44.37%)
female	247 (56.52%)	415 (55.63%)
Onset age (mean age ± SD)	21.25 ± 12.53 yrs	NA
Duration of disease (mean ± SD)	7.8 ± 6.9 yr	NA
Family history	57 (13.04%)	NA

5.2.2 Genomic DNA Preparation:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

5.2.3 Determination of *CTLA4* gene polymorphisms:

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to genotype exon 1 +49A/G and 3' UTR CT60A/G polymorphisms of *CTLA4* gene. The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 30 cycles of 95°C for 30 seconds, primer dependent annealing for 30 seconds (Table 2), and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes (New England Biolabs, Beverly, MA) used were: *BstE*II and *Nco*I for digesting amplicons of exon 1 +49A/G and 3' UTR CT60A/G of *CTLA4* gene. 5 µL of the amplified products were digested for 16 hours at 37°C with 1 U of the corresponding restriction enzyme in a total reaction volume of 25 µL. The digestion products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved in 3.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator.

Table 2. Primers used for *CTLA4* SNPs genotyping and gene expression analysis.

Gene/SNP	Primer Sequence	Annealing Temperature	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs231775)				
CTLA4 +49A/G F	5'-AAGGCTCAGCTGAACCTGGT-3'	60°C	271	*BstE*II (249 & 22 bp)
CTLA4 +49A/G R	5'-CTTTGCCTTATTTGCTGCCGC-3'			
(rs3087243)				*Nco*I
CTLA4 CT60A/G F	5'-CACCACTATTTGGGATATACC-3'	63°C	216	(174 & 42 bp)
CTLA4 CT60A/G R	5'-AGCTCTATATTTCAGGAAGGC-3'			
flCTLA-4 gene expression F	5'-TATGTAATTGATCCAGAACCGTGC3'	63°C	123	-
flCTLA4 gene expressionR	5'-TAGCATTTTGCTCAAAGAAACAG-3'			
sCTLA4 gene expression F	5'GAACCCAGATTTATGTAATTGCTAAG3'	63°C	88	-
sCTLA4 gene expressionR	5'-CACATTCTGGCTCTGTTGGG-3'			
GAPDH gene expression F	5'-ATCCCATCACCATCTTCCAGGA-3'	63°C	122	-
GAPDH gene expressionR	5'-CAAATGAGCCCCAGCCTTCT-3'			

5.2.4 Determination of s*CTLA*4, fl*CTLA*4 and *GAPDH* mRNA expression:

5.2.4.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure™- blood Kit (Ambion inc. Texas,USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis/ ethidium bromide staining and O.D. 260/280 absorbance ratio >1.95. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

5.2.4.2 Real-time PCR:

The levels of full length, soluble *CTLA*4 and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 15 s, 63°C for 30 s, 72°C for 30 s). The fluorescent data collection was performed during the extension step (Figure 7A). At the end of the amplification phase a melt curve analysis was carried out on the product formed (Figure 7B). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

5.2.5 Statistical analysis:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for both the polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the

genotypes and allele frequencies of *CTLA*4 +49A/G and CT60A/G polymorphisms for patients and control subjects were compared using the chi-square test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003).

Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi *et al.,* 2005). The linkage disequilibrium (LD) coefficients D' = D/Dmax and r^2-values for the pair of the most common alleles at each site were estimated using the Haploview programe version 4.1 (Barrett *et al.,* 2005). Differences were considered to be statistically significant if the *p*-value was ≤ 0.05. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Relative expression of both full length *CTLA*4 (flCTLA4) and soluble *CTLA*-4 (s*CTLA*4) in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al.,* 2007).

5.3 RESULTS

5.3.1 Analysis of association between *CTLA*4 gene exon 1 +49A/G polymorphism and susceptibility to vitiligo:

PCR-RFLP for +49A/G polymorphism yielded a 271 bp undigested product corresponding to G allele and 249 bp and 22 bp digested products corresponding to A allele. The three genotypes identified by 3.5% agarose gel electrophoresis were: AA homozygous, AG heterozygous and GG homozygous for +49A/G polymorphism of *CTLA*4 gene (Figure 1A).

Exon 1 +49A/G polymorphism of *CTLA*4 gene was not found to be associated with vitiligo patients (p=0.771) when genotypes were compared with chi-square test-3x2 contingency table (Table 3). Also, there was no significant difference in allele frequencies of this polymorphism between patients and controls when compared with 2x2 contingency table (p=0.461) (Table 3). Both patient and control populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.272 and p=0.126 respectively) (Table 3). This study has 95% statistical power for the effect

size 0.1 to detect association of +49A/G polymorphism of *CTLA*-4 at $p<0.05$ in patients and control population.

5.3.2 Analysis of association between 3' UTR *CTLA*4 gene CT60A/G polymorphism and susceptibility to vitiligo:

The genotyping of CT60A/G polymorphism revealed a 216 bp undigested product corresponding to G allele and 174 bp and 42 bp digested products corresponding to A allele by PCR-RFLP method. The three genotypes identified by 3.5% agarose gel electrophoresis were: AA homozygous, AG heterozygous and GG homozygous for CT60A/G polymorphism of *CTLA*4 gene (Figure 1B).

The 3' UTR CT60A/G polymorphism of *CTLA*4 gene was found to be in significant association with vitiligo patients ($p=0.0002$) when genotypes were compared using chi-squared test-3x2 contingency table with Bonferroni's correction (Table 3). Also, there was significant difference in allele frequencies of this polymorphism between patients and controls when compared with 2x2 contingency table ($p=0.0002$) (Table 3). Control population was found to be in Hardy-Weinberg equilibrium for this polymorphism however patient population deviated from the equilibrium ($p=0.075$ and $p<0.0001$ respectively) (Table 3). This study has 96% statistical power for the effect size 0.1 to detect association of CT60A/G polymorphism of *CTLA*4 at $p<0.05$ in patients and control population.

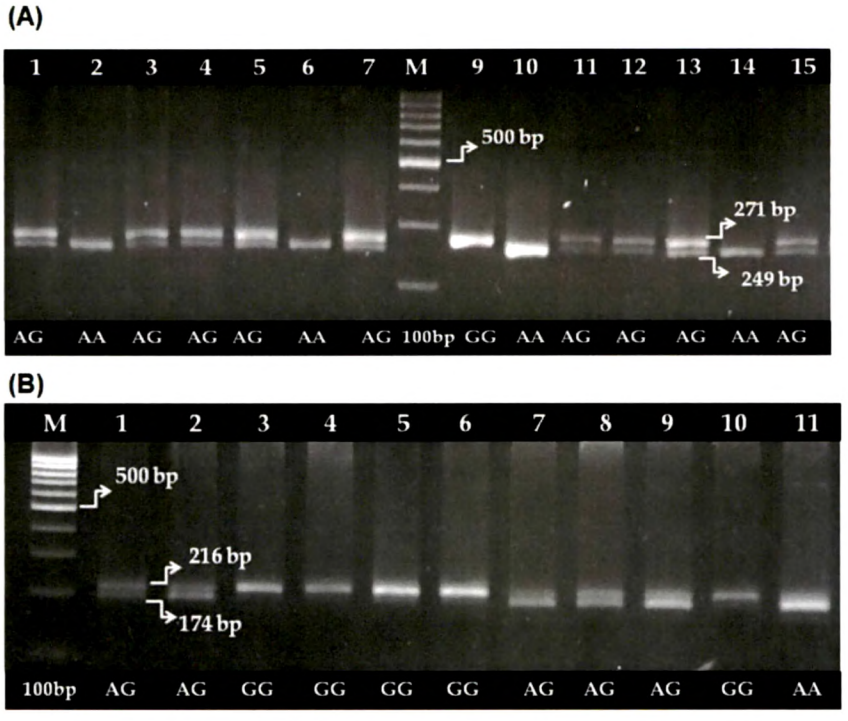


Figure 1. PCR-RFLP analysis of *CTLA*4 exon 1 +49 A/G and3' UTR CT60A/G polymorphisms:

(A) PCR-RFLP analysis of *CTLA*4 exon 1 +49 A/G polymorphism on 3.5 % agarose gel electrophoresis: lanes: 1, 3, 4, 5, 7, 11, 12, 13 & 15 show heterozygous (AG) genotypes; lanes: 2, 6, 10 & 14 show homozygous (AA) genotypes; lane: 9 shows homozygous (GG) genotype; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *CTLA*4 3' UTR CT60A/G polymorphism on 3.5 % agarose gel electrophoresis: lanes: 1, 2, 7, 8 & 9 show heterozygous (AG) genotypes; lanes: 3, 4, 5, 6, & 10 show homozygous (GG) genotypes; lane: 11 shows homozygous (AA) genotype; lane M shows 100 bp DNA ladder.

Table 3. Association studies for *CTLA4* gene exon 1 +49A/G and 3' UTR CT60A/G polymorphisms in generalized vitiligo patients.

SNP	Genotype or allele	Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n =347)	(n =746)			
	AA	169 (0.49)	347 (0.47)			
rs231775	AG	140 (0.40)	310 (0.41)	0.771		
Exon 1	GG	38 (0.11)	89 (0.12)		0.272	
(+49A/G)	Allele				(P)	0.930
	A	478 (0.69)	1004 (0.67)	0.461		(0.766-
	G	216 (0.31)	488 (0.33)		0.126	1.128)
					(C)	
	Genotype	(n = 437)	(n = 738)			
	AA	146 (0.33)	256 (0.35)			
rs3087243	AG	135 (0.31)	337 (0.46)	0.0002*		
3' UTR	GG	156 (0.36)	145 (0.19)		<0.0001	
(CT60A/G)	Allele				(P)	1.417
	A	427 (0.49)	849 (0.58)	0.0002*	0.075	(1.198-
	G	447 (0.51)	627 (0.42)		(C)	1.677)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

(P) refers to Patients and (C) refers to Controls

* Bonferroni's corrected *p* value.

5.3.3 Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the two polymorphisms investigated in the *CTLA4* gene were in moderate LD association (+49A/G: CT60A/G; D' =0.64, r^2=0.11). A haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes did not differ between vitiligo patients and controls (global *p*-value = 0.123) (Table 4). However, the GG haplotype was more frequently observed in vitiligo patients and increased the risk of vitiligo by 1.2-fold [*p* = 0.048; odds ratio (OR): 1.243; 95% confidence interval (CI): (1.001-1.543)] (Table 4).

Table 4. Distribution of haplotypes frequencies for *CTLA4* gene polymorphisms (+49A/G and CT60A/G) among generalized vitiligo patients and controls.

Haplotype (+49A/G and CT60A/G)	Patients (Freq. %) (n=654)	Controls (Freq. %) (n=1374)	*p* for Association	$p_{(global)}$	Odds ratio (95% CI)
AA	178.29 (27.3)	388.84 (28.3)	0.660		0.951 (0.758-1.191)
AG	218.71 (33.4)	523.49 (38.1)	0.061	0.123	0.817 (0.661-1.009)
GA	30.71 (4.7)	52.21 (3.8)	0.372		1.256 (0.761-2.071)
GG	226.29 (34.6)	10.83(29.8)	0.048		1.243 (1.001-1.543)

5.3.4 The expression of fl*CTLA4* and s*CTLA4* transcripts:

Comparison of the findings shows significantly decreased expression of both full length and soluble *CTLA4* in vitiligo patients than in unaffected controls after normalization with *GAPDH* expression (p=0.007 and p=0.037 respectively) (Figure 2A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately two fold change in the expression of fl*CTLA4* and s*CTLA4* mRNA expression in patients as compared to controls (Figure 2B).

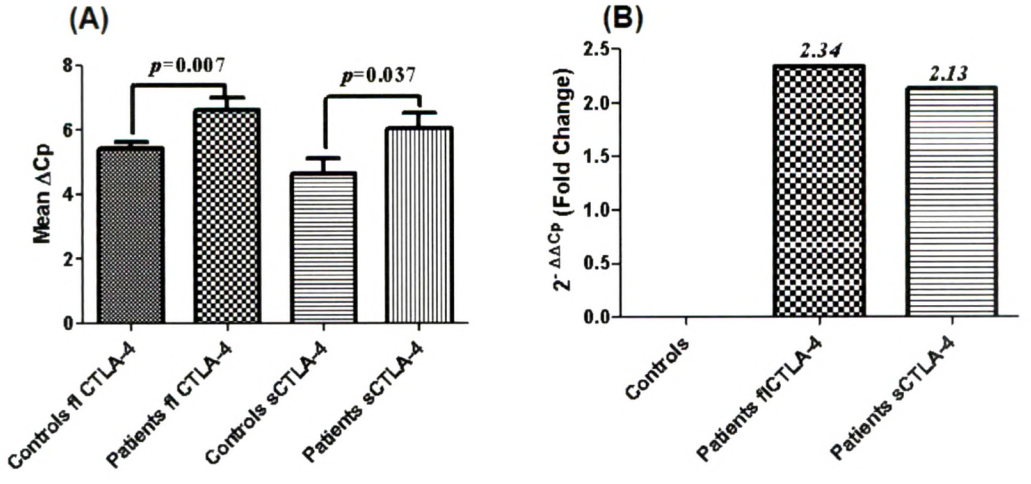


Figure 2. Relative gene expression of fl*CTLA*4 and s*CTLA*4 in controls and vitiligo patients:

(A) Expression of fl*CTLA*4 and s*CTLA*4 mRNA in 76 vitiligo patients and 83 controls as suggested by Mean ΔCp. Vitiligo patients showed significantly reduced mRNA levels of fl*CTLA*4 (p=0.007) and s*CTLA*4 (p=0.037) as compared to controls.

(B) Expression fold change of fl*CTLA*4 and s*CTLA*4 in 76 vitiligo patients against 83 controls showed approximately two fold change as determined by $2^{-\Delta\Delta Cp}$ method.

5.3.5 Genotype-phenotype correlations for s*CTLA*4 and fl*CTLA*4 in vitiligo patients and controls:

We analyzed the mRNA expression of fl*CTLA*4, s*CTLA*4 based on the +49A/G and CT60A/G genotypes of 76 vitiligo patients and 83controls. The expression levels of fl*CTLA*4 for AA, AG and GG genotypes of exon 1 +49A/G polymorphism did not differ significantly in vitiligo patients as compared to controls (p=0.122, p=0.320 and p=0.068 respectively) (Figure 3A). Also, s*CTLA*4 expression did not differ for AA, AG and GG genotypes of exon 1 +49A/G polymorphism in vitiligo patients as compared to controls (p=0.877, p=0.437 and p=0.360 respectively) (Figure 3B). However, the expression levels of both fl*CTLA*4 and s*CTLA*4 were differed significantly for GG genotype of CT60A/G polymorphism in vitiligo patients as compared to controls (p=0.004 and p=0.005 respectively) (Figure 4A & 4B). The AA genotype did not differ for fl*CTLA*4 and s*CTLA*4 expression levels in patients and controls (p=0.343 and p=0.205 respectively) (Figure 4A & 4B).

Further, the expression levels of both fl*CTLA*4 and s*CTLA*4 were analyzed with respect to haplotypes generated from the two investigated polymorphisms of *CTLA*4 (Fig. 4A & 4B). Both fl*CTLA*4 and s*CTLA*4 expression levels were significantly differed and associated with AG haplotypes in patients and controls (p=0.036 and p=0.020 respectively). Other, three haplotypes: AA, GA and GG did not differ with respect to fl*CTLA*4 and s*CTLA*4 expression levels in patients and controls (p=0.955 & p=0.152, p=0.476 & p=0.865, p=0.075 & p=0.992 respectively).

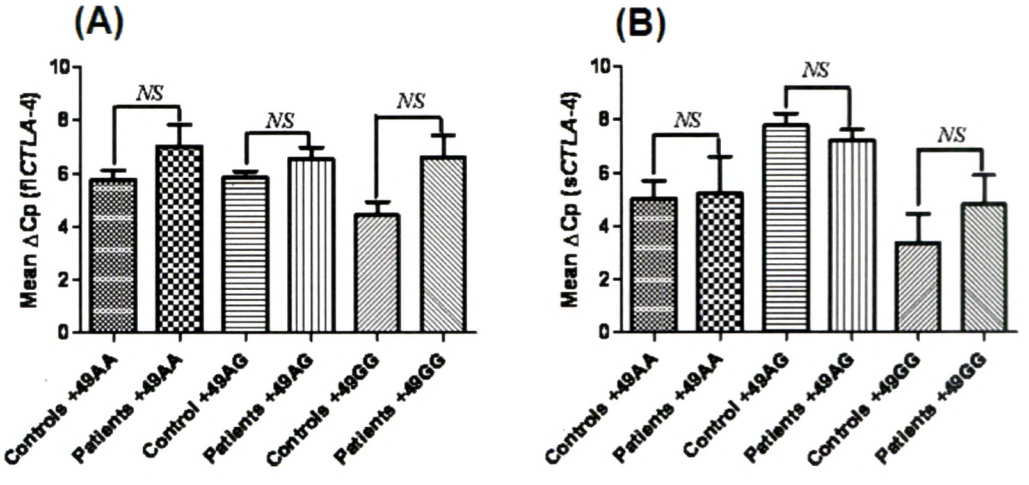


Figure 3. Genotype - phenotype correlation of exon 1 +49A/G polymorphism of fl*CTLA*4 and s*CTL*A4 in controls and vitiligo patients:

(A) Relative mRNA expression of *flCTLA4* with respect to +49A/G genotypes in 76 patients and 83 controls. None of the three genotypes AA (p=0.122), AG (p=0.320) and GG (p=0.068) in patients showed significant difference for *flCTLA*4 expression as compared to controls a suggested by Mean ΔCp.

(B) Relative mRNA expression of *sCTLA4* with respect to +49A/G genotypes in 76 patients and 83 controls. None of the three genotypes AA (p=0.877), AG (p=0.437) and GG (p=0.360) in patients showed significant difference for *flCTLA4* expression as compared to controls a suggested by Mean ΔCp.

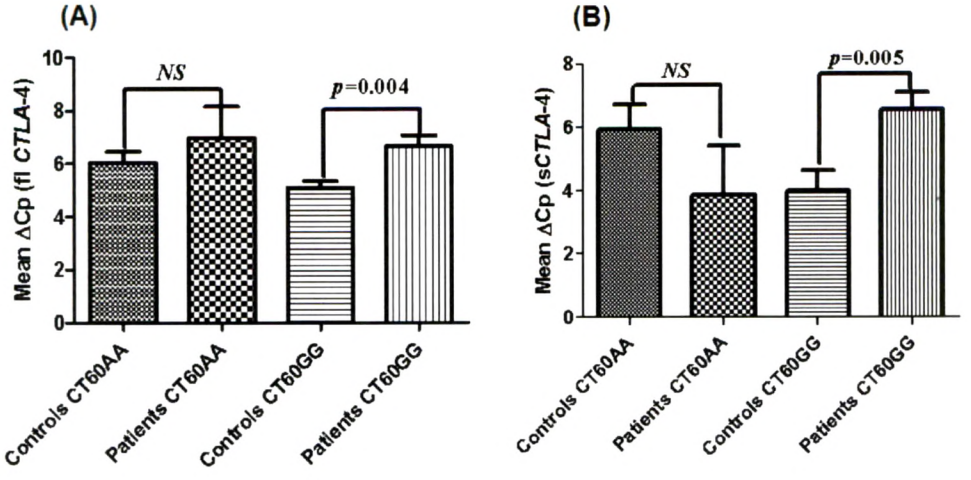


Figure 4. Genotype - phenotype correlation of 3' UTR CT60A/G polymorphism of *flCTLA4* and *sCTLA4* in controls and vitiligo patients:

(A) Relative mRNA expression of *flCTLA4* with respect to CT60A/G genotypes in 76 patients and 83 controls. GG genotype showed significant decrease in the levels of *flCTLA4* mRNA (p=0.004) in patients as compared to AA genotype (p=0.343) as suggested by Mean ΔCp.

(B) Relative mRNA expression of *sCTLA4* with respect to CT60A/G genotypes in 76 patients and 83 controls. GG genotype showed significant decrease in levels of *sCTLA4* mRNA (p=0.005) in patients as compared to AA genotype (p=0.205) as suggested by Mean ΔCp. [NS = non-significant]

5.3.6 Ratio of *sCTLA4* and *flCTLA4* mRNA expression in vitiligo patients and controls:

The expression level of *sCTLA4* and *flCTLA4* was also analyzed as ratio of *sCTLA4*: *flCTLA4* in vitiligo patients and controls. We could not detect any significant difference in the ratio of *sCTLA4* to *flCTLA4* mRNA expression between patients and controls (p=0.346) (Figure 5A). However, AG genotype of exon 1 +49A/G polymorphism showed significant difference for the ratio of *sCTLA4* and *flCTLA4* mRNA expression in patients and controls (p=0.049) (Figure 6A). Whereas the other two genotypes: AA and GG did not differ for the ratio of *sCTLA4* and *flCTLA4* mRNA expression in patients and controls (p=0.668 and p=0.964) (Figure 6A). When ratio of *sCTLA4* and *flCTLA4* mRNA expression for CT60A/G genotypes were

compared in patients and controls GG genotype showed significantly decreased ratio (p=0.019), conversely the AA genotype did not differ significantly (p=0.096) (Figure 6B). Moreover, ratio of *sCTLA4* and *flCTLA4* mRNA expression did not differ significantly for any of the haplotypes (AA, AG, GA, GG) in patients and controls (p=0.159, p=0.068, p=0.966 and p=0.585 respectively) (Figure 5B).

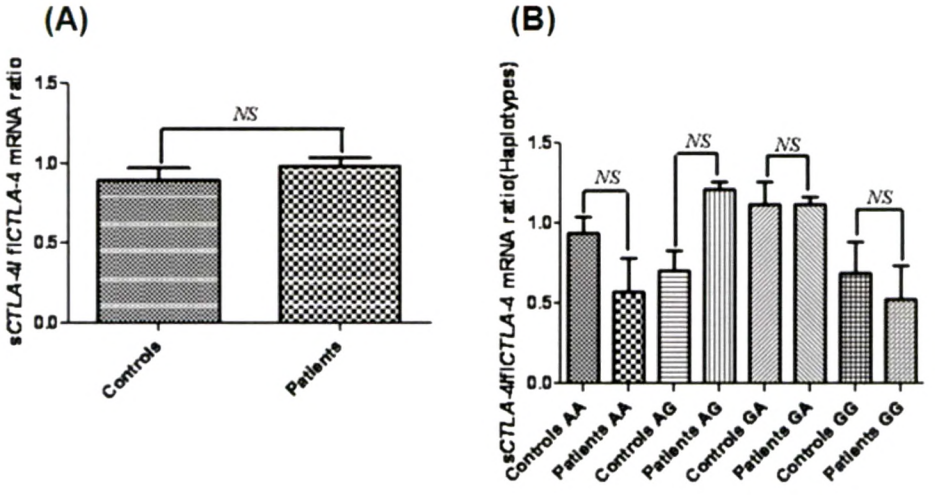


Figure 5. Expression of human *sCTLA4* and *flCTLA4* mRNA ratio in controls and vitiligo patients:

(A) *sCTLA4/flCTLA4* mRNA ratio was measured in 76 vitiligo patients and 83 unaffected controls which was not found altered (p=0.346).

(B) *sCTLA4/flCTLA4* mRNA ratio was analyzed with respect to (+49A/G: CT60A/G) haplotypes in patients and controls. None of the haplotypes showed significant difference in the *sCTLA4/flCTLA4* mRNA ratio (AA, AG, GA, GG) in patients and controls (p=0.159, p=0.068, p=0.966 and p=0.585 respectively). [*NS* = non-significant]

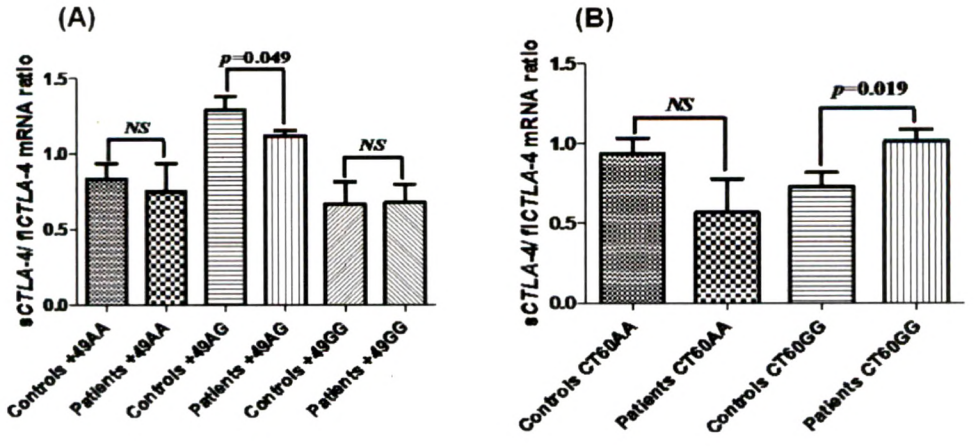
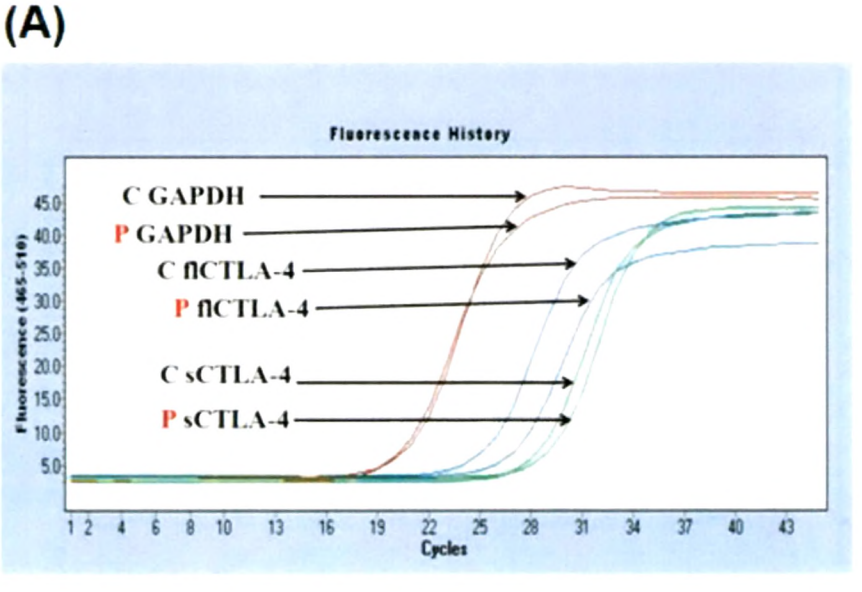


Figure 6. Expression of human *sCTLA4* and *flCTLA4* mRNA ratio with respect to +49A/G and CT60A/G genotypes in controls and patients:

(A) *sCTLA4/flCTLA4* mRNA ratio was analyzed with respect to +49A/G genotypes in patients and controls. AG genotype showed significant decrease in the ratio (p=0.049) as compared to AA (p= 0.668) and GG (p=0.964) genotypes.

(B) s*CTLA4/flCTLA4* mRNA ratio was analyzed with respect to CT60A/G genotypes in patients and controls. GG genotype showed significant increase in the ratio (p=0.019) as compared to AA (p=0.096) genotype.



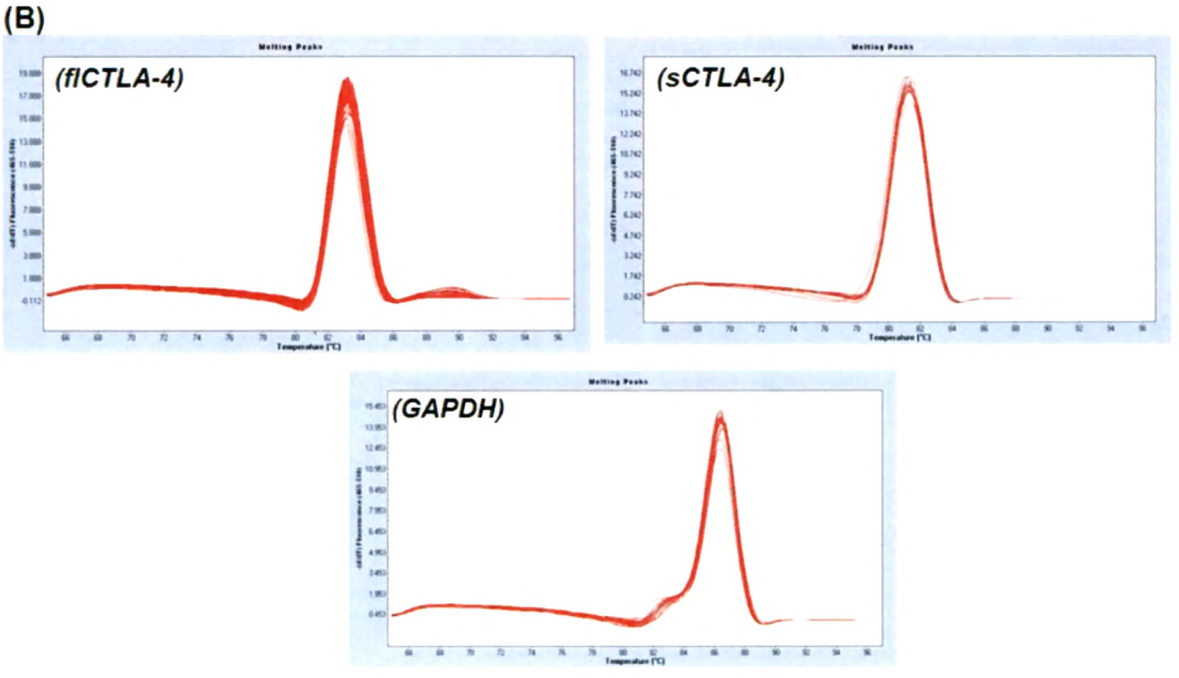


Figure 7. Amplification curve for relative gene expression of *flCTLA4* and *sCTLA4*:

(A) Representative amplification curve for *flCTLA*4 and *sCTLA4* with *GAPDH* as reference gene. 'C' refers to Control and 'P' refers to Patient. **(B)** Melt curve analysis of *flCTLA4*, *sCTLA4* and *GAPDH* showing specific amplification.

5.4 DISCUSSION

Vitiligo cannot be explained by simple Mendelian genetics, however it is characterized by incomplete penetrance, multiple susceptibility loci and genetic heterogeneity (Zhang *et al.,* 2005). Attempts to identify genes involved in susceptibility to vitiligo have involved gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses to discover new genes. Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo since it shows frequent association with other autoimmune disorders such as rheumatoid arthritis, Graves' disease etc. (Ochi *et al.,* 1969). About 25-30% of generalized vitiligo patients manifest at least one other autoimmune disease; particularly Graves' disease, autoimmune hypothyroidism, rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, Addison's

disease, and systemic lupus erythematosus (Alkhateeb *et al.,* 2003; Laberge *et al.,* 2005). Our study also suggested that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil *et al.,* 2006b). Also, in the present study 13.04% of vitiligo patients have one or more first degree relative affected suggesting the involvement of genetic factors in pathogenesis of vitiligo.

CTLA-4 is an important molecule in the down-regulation of T-cell activation and it is critical for the activity of regulatory T cells. The CTLA4 gene produces two different isoforms: a full length protein (flCTLA4) encoded by exon 1 (peptide leader), exon 2 (extracellular domain), exon 3 (transmembrane domain) and exon 4 (cytoplasmic domain); and a soluble form (sCTLA4) in which exon 3 is lost (Teft *et al.,* 2006). The soluble form is expressed by resting T cells and its expression disappears within 48 h of activation (Oaks *et al.,* 2000). In activated T cells, the flCTLA4 serves as a transmembrane receptor to inhibit cell proliferation. By contrast, the role of sCTLA4 is not yet known, although it has been shown to act as a functional receptor for B7 antigens, suggesting that sCTLA4 can block the B7-CD28 interaction and interfere with the co-stimulation signal, inhibiting T-cell proliferation (Teft *et al.,* 2006).

Recent studies have shown that the function of regulatory T cells is impaired in autoimmune diseases. The lack of *CTLA4* in knockout mice produces important lymphoproliferative disorders, multi-organ destruction and premature death (Waterhouse *et al.,* 1995). Moreover, *CTLA4* siRNA knock-down in animals leads to the development of autoimmune diabetes (Chen *et al.,* 2006).

In the present study we analyzed the association of two polymorphic sites in the *CTLA4* gene i.e., exon 1: +49A/G and in the 3' UTR region: CT60A/G with vitiligo susceptibility in Gujarat population. We report an association between CT60A/G polymorphism and susceptibility to vitiligo in the Gujarat population. In particular, we found that the presence of CT60GG genotype was more prevalent among vitiligo patients. On contrary, studies from Caucasian and Romanian populations did not find association of CT60A/G polymorphism with vitiligo (LaBerge *et al.,* 2008; Birlea *et al.,* 2009). Another study from Caucasian population showed significant association of the polymorphism only with vitiligo patients having other autoimmune diseases however, this study involved very less sample size (Blomhoff *et al.,* 2005). We did

not find any significant association of genotype and allele frequencies for +49A/G polymorphism in vitiligo patients and controls. In addition, studies from South Indian, Iranian and Romanian populations did not show association of +49A/G polymorphism in vitiligo patients (Deeba *et al.,* 2010; Fattahi *et al.,* 2005; Birlea *et al.,* 2009).

Recently, Birlea et al. (2011) analyzed 104 SNPs in *CTLA4* gene and found one promoter SNP (rs12992492) showing maximum association with both generalized vitiligo (GV) patients and patients having other autoimmune diseases; however patients with isolated GV without other autoimmune diseases did not achieve the study significance threshold for any other SNPs in *CTLA4* gene. This study together with other previous observations suggests that association of *CTLA*4 with GV is secondary, driven by primary genetic association of *CTLA*4 with other autoimmune diseases that are associated with vitiligo. However, the present study did not include vitiligo patients having other autoimmune diseases and surprisingly this study found the significant association of CT60A/G polymorphism with vitiligo patients having no other autoimmune diseases. The deviation of the study from the previous reports may be due to ethnic differences.

Furthermore, to evaluate the possible expression dysregulation of *CTLA*4 isoforms important in T regulatory cell's function, the transcript levels of fl*CTLA*4 and s*CTLA*4 genes were measured in patients with vitiligo and compared with those from unaffected subjects. This is the first report where *CTLA*-4 expression levels were studied in patients with vitiligo. Interestingly, we found decreased mRNA expression of both fl*CTLA*4 and s*CTLA*4 in vitiligo patients as compared to controls. We further analyzed whether the polymorphisms examined in this study influenced the expression levels of fl*CTLA*4 and s*CTLA*4. The CT60A/G dimorphism has been postulated to affect alternative splicing of the *CTLA*4 gene, resulting in decreased levels of soluble CTLA4 (sCTLA4) mRNA which is in concordance with our results (Ueda *et al.,* 2003). Recently, Gerold et al. (2011) has also shown decreased s*CTLA*4 levels in Type-1 diabetic condition and suggested that lower s*CTLA*4 expression may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. In contrast, increased serum sCTLA4 levels were detected in other autoimmune diseases such as Graves' disease (Daroszweski *et al.,* 2009), autoimmune thyroid disease (Saverino 2007), psoriasis (Luszczek *et al.,* 2006) and Crohn's disease (Chen

et al., 2011). These studies suggest that s*CTLA*4 may contribute to the development of autoimmune diseases, probably through inhibiting the B7-flCTLA4 interaction and down-regulation of T cell activation.

In present study the +49A/G genotypes did not influence fl*CTLA*4 and s*CTLA*4 mRNA expression suggesting that it could not contribute major role in vitiligo susceptibility of Gujarat population. Nevertheless, its associations with other diseases are also controversial. Previously, the +49A/G variation was found to affect the cell surface expression of *CTLA*4 but did not affect the expression in other study (Anjos *et al.,* 2002; Munthe-Kaas *et al.,* 2004).

The 3' UTR of *CTLA*4 gene has been found to be involved in several autoimmune diseases, hence to study the genetic variation of such regions is imperative. Interestingly, 3' UTR CT60G allele greatly reduced the mRNA expression of both fl*CTLA*4 and s*CTLA*4 in vitiligo patients as compared to controls suggesting its crucial role in pathogenesis of vitiligo. Further, the haplotype AG (+49A:CT60G) greatly decreased mRNA levels of s*CTLA*4 in patients than fl*CTLA*4 as compared to controls revealing the positive correlation of CT60G in vitiligo pathogenesis.

Moreover, s*CTLA*4/fl*CTLA*4 mRNA ratio was found to be increased with +49AG and CT60GG genotype in vitiligo patients as compared to AA genotypes suggesting G allele to confer lower mRNA levels of s*CTLA*4. Atabani et al. (2005) also found elevated sCTLA4 to flCTLA4 mRNA expression with the CT60 GG and AA genotypes. However, Anjos et al. (2005) and Mayans et al. (2007) could not find effect of the CT60 polymorphism on the expression of s*CTLA*4 and fl*CTLA*4. The present study found higher prevalence of CT60GG genotypes among vitiligo patients however, CT60AA genotype does not seem to modulate *CTLA*4 mRNA expression and hence patients harboring it may have other genetic factors involved in disease pathogenesis supporting the fact that vitiligo may have varied type of precipitating factors. In addition, patients harboring CT60GG genotype had reduced levels of *CTLA*4 mRNA as compared to controls suggestive of CT60GG genotype having a profound effect on *CTLA*4 in progression of disease.

CTLA4 may modulate immune response in two ways: as a cell membrane receptor transmitting a negative signal to the T cell upon ligand binding and as a soluble

molecule that may bind to the same ligand, preventing in this way the positive stimulation of the T cell via CD28 or the negative signaling via cell membrane CTLA4 (Teft *et al.,* 2006; Pawlak *et al.,* 2005). These different interactions may, in part, be responsible for the contradictory results of previous studies of associations of *CTLA*4 polymorphisms with autoimmune diseases. Therefore, further research on *CTLA*4 polymorphisms, dynamics of full length versus s*CTLA*4 expression and turnover in vitiligo as well as, other autoimmune diseases should clarify the role of *CTLA*4 in the regulation of immune response in physiological and pathological situations.

In conclusion, our findings suggest that the dysregulated *CTLA*4 expression in vitiligo patients could result, at least in part, from variations at the genetic level and this study has been published [Dwivedi M, Laddha NC, Imran M, Shah BJ and Begum R (2011). Cytotoxic T-lymphocyte associated antigen-4 (CTLA4) in isolated vitiligo: a genotype-phenotype correlation. Pigment Cell Melanoma Res. 24: 737-740]. For the first time, we show that the 3' UTR CT60A/G polymorphism of the *CTLA*4 gene influences both full length and soluble *CTLA*4 mRNA levels in vitiligo patients, and thus this genotype-phenotype correlation of *CTLA*4 supports the autoimmune pathogenesis of vitiligo.

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CHAPETR VI

MELANOCYTE PROLIFERATING GENE 1 (*MYG1*) PROMOTER AND STRUCTURAL POLYMORPHISMS AND THEIR GENOTYPE-PHENOTYPE CORRELATION WITH VITILIGO SUSCEPTIBILITY

6.1 INTRODUCTION

Vitiligo is an acquired hypomelanotic disease characterized by circumscribed depigmented macules. Absence of melanocytes from the lesional skin due to their destruction has been suggested to be the key event in the pathogenesis of vitiligo (Ortonne and Bose, 1999). It is frequently associated with a positive family history and affects approximately 0.5-1% of the world population (Taieb, 2007). In India, the incidence of vitiligo is found to be 0.5-2.5% wherein Gujarat and Rajasthan states have the highest prevalence upto ~8.8% (Handa and Kaur, 1999). Autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity (Shajil *et al.*, 2006; Kemp *et al.*, 2001). The autoimmune hypothesis gains further support from immunotherapy studies of melanoma patients (Rosenberg, 1997).

Vitiligo is a polygenic disease and attempts to identify genes involved in susceptibility include gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses to discover new genes. Recent genome-wide association studies (GWAS), have identified generalized vitiligo susceptibility genes which involve immune regulation and immune targeting of melanocytes, suggesting that generalized vitiligo is a primary autoimmune disease however, the biological triggers of the autoimmune process remain unknown (Spritz, 2011). Several candidate genes have been tested for genetic association with generalized vitiligo, including the *MHC*, *ACE*, *CAT*, *CTLA*-4, *COMT*, *ESR*, *GCH*1, *MBL2*, *PTPN*22, and *VDR* (Spritz, 2007 & 2008). Most of these studies reported significant associations, although some yielded only marginal significance and several were not replicated by subsequent studies. Recently, a number of genes which play a role in vitiligo susceptibility, including *HLA*, *PTPN*22, *NALP*1, *XBP*1, *FOXP*1, *IL2RA* have been tested for genetic association with vitiligo (Spritz, 2010). *MYG1* [Melanocyte proliferating gene 1, C12orf10 in human] is a recently described gene of unknown function. According to one study *MYG1* is a highly expressed gene in freely proliferating melanocytes and down regulated in malignant melanoma cells and suggesting that *MYG1* could be a melanocyte specific gene (Smicun, 2000).

MYG1 is a ubiquitous nucleo-mitochondrial protein involved in early developmental processes (Philips *et al.*,2009); however *MYG1* expression in normal adult tissues is stable and seems to be changed mainly as a response to stress/illness conditions (Kingo *et al.*,2006; Hawse *et al.*, 2003; Kõks *et al.*,2004). MYG1 is the only member of an uncharacterized protein family UPF0160 (PF03690) and contains a number of metal binding residues and is predicted to possess metal-dependent protein hydrolase activity. MYG1 protein is highly conserved and present in all eukaryotes from yeast to humans (Nepomuceno-Silva *et al.*, 2004).

According to global expression analysis, *MYG1* tends to be up-regulated in undifferentiated and pluripotent cells. Kingo *et al.*, (2006) have suggested the involvement of *MYG1* in vitiligo pathogenesis by showing elevated expression of *MYG1* mRNA in both uninvolved and involved skin of vitiligo patients. In addition, *MYG1* has been found to be consistently up-regulated in skin biopsies from patients with atopic eczema (Sääf *et al.*, 2008).

Recently, Philips *et al.*, (2010) have proposed that *MYG1* gene may be involved in vitiligo pathogenesis. This study demonstrated that both *MYG1* promoter polymorphism -119C/G and Arg4Gln polymorphism in the mitochondrial signal of *MYG1* have a functional impact on the regulation of the *MYG1* gene and promoter polymorphism (-119C/G) is related with susceptibility for actively progressing vitiligo in Caucasian population. Hence, the study has prompted us to investigate the involvement of *MYG1* gene in precipitation of vitiligo in Gujarat population.

The objectives of this study were:

i.) To determine whether *MYG1* promoter (rs1465073) and structural (rs1534284-rs1534283) polymorphisms are associated with vitiligo susceptibility in Gujarat population.

ii.) To measure and compare *MYG1* transcript levels in patients with vitiligo and in unaffected controls.

iii.) To determine genotype-phenotype correlation of *MYG1* with vitiligo susceptibility.

6.2 MATERIALS AND METHODS

6.2.1 Study subjects

The study group included 846 vitiligo patients [668 generalized (including acrofacial vitiligo and vitiligo universalis) and 178 localized vitiligo cases] comprised of 389 males and 457 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune disease. A total of 726 ethnically sex-matched unaffected individuals (347 males and 379 females) were included as controls in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls

	Vitiligo Patients	**Controls**
	(n = 846)	(n = 726)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	389 (45.98%)	347 (47.79%)
Female	457 (54.02%)	379 (52.21%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Type of vitiligo		
Generalized	668 (78.96%)	NA
Localized	178 (21.04%)	NA
Active vitiligo	599 (70.80%)	NA
Stable vitiligo	247 (29.20%)	NA
Family history	116 (13.71%)	NA

6.2.2 Genomic DNA Preparation

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

6.2.3 Determination of *MYG1* promoter and structural polymorphisms

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype promoter and structural polymorphisms of *MYG1* gene (Figure 1A & B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes (New England Biolabs, Beverly, MA) *Hinf*I and *Tas*I were used for digesting amplicons of -119C/G and Arg4Gln (AA/GC) respectively of *MYG1* gene (Table 2). 5 µL of the amplified products were digested with 5 U of the corresponding restriction enzyme in a total reaction volume of 25 µL, according to the manufacturer's instructions. The digested products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were

100% concordant (analysis of the chosen samples was repeated by two researchers independently).

Table 2. Primers and restriction enzymes used for *MYG1* SNPs genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs1465073)				
MYG1 -119C/G F	GGATTAAGCAAGGGACTGGTGATG	60	347	*Hinf*I (150 & 197 bp)
MYG1 -119C/G R	CATGCGGTGCCGGGTATACA			
(rs1534284-rs1534283)				
MYG1 AA/GC F	GGATTAAGCAAGGGACTGGTGATG	63	347	*Tas*I (282 & 65 bp)
MYG1 AA/GC R	CATGCGGTGCCGGGTATACA			
MYG1 expression F	GGAGTACCGGGATGCAGAG	65	290	-
MYG1 expressionR	CATACATCTTGTCATAGAGGGTGC			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expressionR	CAAATGAGCCCCAGCCTTCT			

6.2.4 Determination of *MYG1* and *GAPDH* mRNA expression

6.2.4.1 RNA extraction and cDNA synthesis

Total RNA from whole blood was isolated and purified using Ribopure™- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was

verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

6.2.4.2 Real-time PCR

The expression of *MYG1* and *GAPDH* transcripts were measured by real-time PCR using SYBR Green method and gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 10 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 3E). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

6.2.5 Statistical analyses

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for both the polymorphisms in patients and controls by comparing the observed and expected frequencies of genotypes using chi-squared analysis. Distribution of the genotypes and allele frequencies of -119C/G and Arg4Gln (AA/GC) polymorphisms for patients and control subjects were compared using the chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. *p*-values less than 0.025 were considered as statistically significant due to Bonferroni's correction for multiple

testing. Age of onset analysis and relative expression of *MYG1* in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al.*, 2007).

6.3 RESULTS

6.3.1 Analysis of association between *MYG1* -119C/G polymorphism and susceptibility to vitiligo

PCR-RFLP for *MYG1* -119C/G polymorphism yielded a 347 bp undigested product corresponding to G allele and 197 bp and 150 bp digested products corresponding to C allele. The three genotypes identified by 2.5% agarose gel electrophoresis were: CC homozygous, CG heterozygous and GG homozygous for -119C/G polymorphism of *MYG1* gene (Figure 1A).

The genotype and allele frequencies of the -119C/G polymorphism in 846 vitiligo patients and 726 controls are summarized in Table 3. The *MYG1* -119C/G polymorphism was found to be in significant association with vitiligo patients ($p<0.0001$) when genotypes were compared using chi-squared test-3x2 contingency table (Table 3). The minor allele (G) of the -119C/G was more frequent in the vitiligo group compared to the control group (65.0% versus 57.0%, $p<0.0001$; OR 1.405, 95% CI 1.216 - 1.623) consistent with a susceptibility effect (Table 3). Control population was found to be in Hardy-Weinberg equilibrium for this polymorphism however, patient population deviated from the equilibrium ($p=0.079$ and $p=0.018$ respectively) (Table 3). Moreover, both generalized and localized vitiligo groups showed significant association of -119C/G polymorphism when the genotypes were compared with those of control group ($p=0.0003$ and $p=0.006$ respectively) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized and localized vitiligo groups when the alleles were compared with those of control group ($p<0.0001$ and $p=0.002$ respectively) (Table 4). The distribution of *MYG1* -119C/G genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups ($p>0{\cdot}05$).

In addition, gender based analysis of *MYG1* -119C/G polymorphism suggested significant association of minor -119G allele, consistent with a susceptibility effect with female vitligo patients as compared to male patients (69.0% versus 60.0%, p=0.0003, OR 1.454, 95% CI 1.190-1.777 (Table 5). This study has 93.77% statistical power for the effect size 0.08 to detect the association of investigated polymorphisms at $p<0.05$ in generalized vitiligo patients and control population.

6.3.2 Analysis of association between *MYG1* Arg4Gln (AA/GC) polymorphism and susceptibility to vitiligo

The genotype and allele frequencies of the investigated *MYG1* Arg4Gln (AA/GC) polymorphism in 846 vitiligo patients and 726 controls are summarized in Table 3. PCR-RFLP for *MYG1* Arg4Gln (AA/GC) polymorphism yielded only a 347 bp undigested product corresponding to GC alleles, identified by 2.5% agarose gel electrophoresis (Figure 1B).

The *MYG1* Arg4Gln (AA/GC) polymorphism was not found to be associated with vitiligo susceptibility ($p=1.00$) (Table 3). Interestingly, the polymorphism was found to be monogenic; being only GC alleles presented in control and both the patient groups Table 3. Also, the distribution of genotype frequencies for *MYG1* promoter polymorphism was not consistent with Hardy-Weinberg expectations in both patient and control groups ($p<0.0001$).

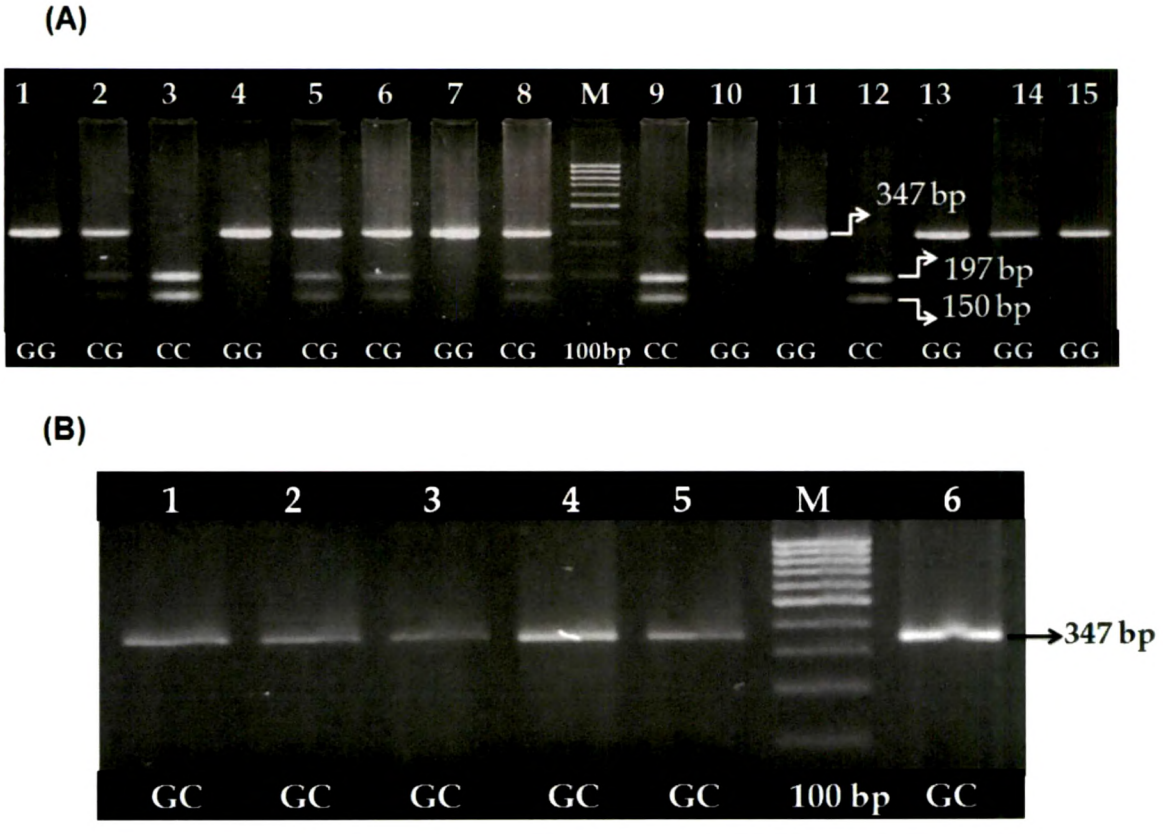

Figure 1. PCR-RFLP analysis of *MYG1* -119C/G and 11-12 AA/GC polymorphisms:

(A) PCR-RFLP analysis of *MYG1* -119C/G polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 4, 7, 10, 11, 13, 14 &15 show homozygous (GG) genotypes; lanes: 2, 5, 6, & 8 show heterozygous (CG) genotypes; lanes: 3, 9 & 12 show homozygous (CC) genotypes; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *MYG1* 11-12 AA/GC polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 2, 3, 4, 5 & 6 show individuals with GC alleles; lane M shows 100 bp DNA ladder.

Table 3. Association studies for *MYG1* gene -119C/G promoter and 11-12AA/GC structural polymorphisms in vitiligo patients from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 846)	(n = 726)			
	CC	120 (0.14)	147 (0.20)		0.018[a]	
rs1465073	CG	354 (0.42)	333 (0.46)	<0.0001	(P)	
Promoter	GG	372 (0.44)	246 (0.34)			
(-119C/G)	Allele				0.079[b]	1.405
	C	594 (0.35)	627 (0.43)	<0.0001	(C)	(1.216-
	G	1098 (0.65)	825 (0.57)			1.623)
	Genotype	(n = 846)	(n = 726)			
	AA	0 (0.00)	0 (0.00)		<0.0001[a]	
rs1534284-	AG	0 (0.00)	0 (0.00)	1.000	(P)	
rs1534283	GC	846 (1.00)	726 (1.00)			
	Allele					1.000
(AA/GC;	G	423 (0.50)	363 (0.50)	1.000	<0.0001[b]	(0.801-
	C	423 (0.50)	363 (0.50)			1.235)
Arg4Gln)					(C)	

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 4. Association studies for *MYG1* gene -119C/G promoter and 11-12AA/GC structural polymorphisms in generalized and localized vitiligo patients from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo Patients (Freq.)	Localized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 668)	(n = 178)	(n = 726)			0.7212
	CC	94 (0.14)	26 (0.15)	147 (0.20)	0.0003[a]	0.082	(0.6189-
rs1465073	CG	285 (0.43)	69 (0.39)	333 (0.46)		(GV)	0.8403)
Promoter	GG	289 (0.43)	83 (0.46)	246 (0.34)	0.006[b]		(GV)
(-119C/G)	Allele					0.069	
	C	473 (0.35)	121(0.34)	627 (0.43)	<0.0001[c]	(LV)	0.6818
	G	863 (0.65)	235(0.66)	825 (0.57)			(0.5349-
					0.002[d]	0.079	0.8692)
						(C)	(LV)
	Genotype	(n = 668)	(n = 178)	(n = 726)			
	AA	0 (0.00)	0 (0.00)	0 (0.00)		<0.00	
rs1534284-	AG	0 (0.00)	0 (0.00)	0 (0.00)	1.000	01	1.000
rs1534283	GC	668 (1.00)	178(1.00)	726 (1.00)	(GV, LV)	(GV,	(0.801-
(AA/GC;	Allele					LV)	1.235)
Arg4Gln)	G	334 (0.50)	89 (0.50)	363 (0.50)	1.000		(GV,
	C	334 (0.50)	89 (0.50)	363 (0.50)	(GV, LV)	<0.00	LV)
						01	
						(C)	

'n' represents number of Patients/ Controls,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval,
(GV) refers to Generalized Vitiligo, (LV) Localized Vitiligo and (C) refers to Controls,
[a] Generalized Vitiligo vs. Controls using chi-squared test with 3×2 contingency table,
[b]Localized Vitiligo vs. Controls using chi-squared test with 3×2 contingency table,
[c] Generalized Vitiligo vs. Controls using chi-squared test with 2×2 contingency table,
[d] Localized Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table,
Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 5. Association studies for *MYG1* gene -119C/G promoter and 11-12AA/GC structural polymorphisms in male and female patients with vitiligo.

SNP	Genotype or allele	Male Patients (Freq.)	Female Patients (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 389)	(n = 457)			
	CC	70 (0.18)	50 (0.11)		0.067	
rs1465073	CG	169 (0.43)	185 (0.40)	0.002[a]	(M)	
Promoter	GG	150 (0.39)	222 (0.49)			
(-119C/G)	Allele				0.225	1.454
	C	309 (0.40)	285 (0.31)	0.0003[b]	(F)	(1.190-
	G	469 (0.60)	629 (0.69)			1.777)
	Genotype	(n = 389)	(n = 457)			
	AA	0 (0.00)	0 (0.00)		<0.0001	
	AG	0 (0.00)	0 (0.00)	1.000	(M)	1.000
rs1534284-	GC	389 (1.00)	457 (1.00)			(0.801-
rs1534283	Allele				<0.0001	1.235)
(AA/GC;	G	389 (0.50)	457 (0.50)	1.000	(F)	
Arg4Gln)	C	389 (0.50)	457 (0.50)			

'n' represents number of Patients,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval,
(M) refers to Male Patients and (F) refers to Female Patients,
[a] Male Patients vs. Female Patients using chi-squared test with 3×2 contingency table,
[b]Male Patients vs. Female Patients using chi-squared test with 2×2 contingency table,
Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

6.3.3 Effect of *MYG1* -119C/G genotypes on age of onset of vitiligo and its progression

When age of onset of the disease was correlated with the *MYG1* -119C/G genotypes, patients with susceptible GG genotypes showed early onset of the disease as compared to CC and CG genotypes (p=0.0002 and p=0.039 respectively) (Figure 2A). Moreover, patients with genotype CG showed early onset of the disease as compared to CC genotypes (p=0.022) (Figure 2A) suggesting the effect of the susceptible allele G on the early onset of disease. Interestingly, when male and female vitiligo patients were analyzed for age of onset of the disease, female patients had significant early onset of the disease as compared to the male patients (p=0.002) (Figure 2B).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the minor -119G allele occurred prevalently in the group of patients with active vitiligo (68.0% versus 57.0%, p<0.0001; OR 0.6207, 95% CI 0.5291-0.7282) consistent with a susceptibility effect compared to the control group (Table 6). However, there was no statistically significant effect in the distribution of the -119G allele between patients with stable vitiligo and control group (p=0.833) (Table 6). Interestingly, the -119G allele was more prevalent in patients with active vitiligo as compared to stable vitiligo (68.0% versus 57.0%, p<0.0001; OR 0.6380, 95% CI 0.5141-0.7916) suggesting the important role of -119G allele in progression of the disease (Table 6).

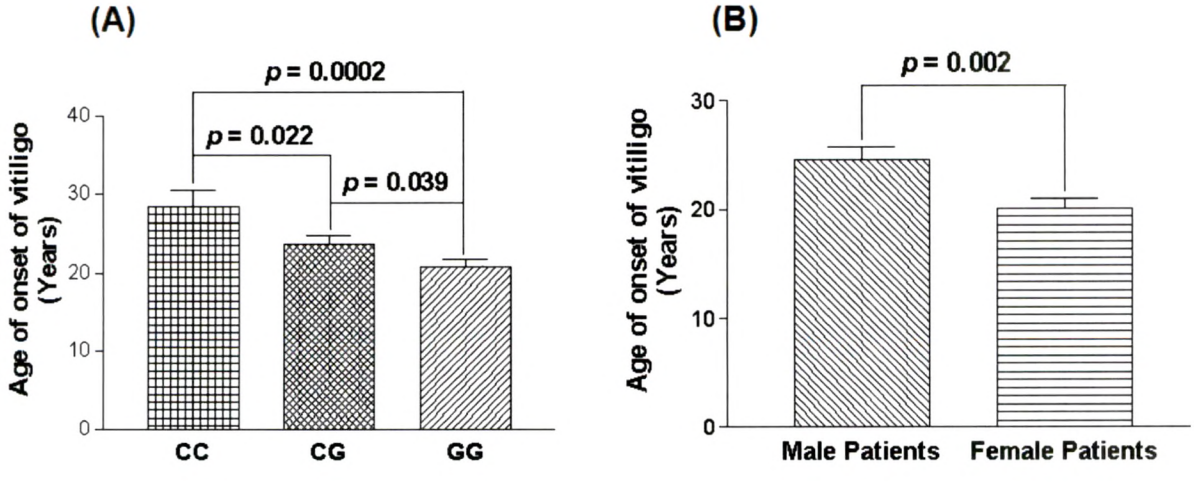

Figure 2. Age of onset of the disease in vitiligo patients:

(A) Comparison of age of onset of the disease (Years) with respect to *MYG1* promoter -119C/G in 846 vitiligo patients.

(B) Comparison of age of onset of the disease (Years) with respect to gender differences in 389 male patients and 457 female patients with vitiligo.

Table 6. Association studies for *MYG1* gene -119C/G promoter and 11-12AA/GC structural polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 599)	(n = 247)	(n = 726)			0.6380[a]
	CC	69 (0.13)	51 (0.17)	147 (0.20)	0.0003[a]	0.162	(0.5141-
rs1465073	CG	246 (0.42)	108 (0.42)	333 (0.46)	<0.0001[b]	(AV)	0.7916)
Promoter	GG	284 (0.45)	88 (0.41)	246 (0.34)	0.833[c]		
(-119C/G)	Allele					0.098	0.6207[b]
	C	384 (0.32)	210 (0.43)	627 (0.43)	<0.0001[a]	(SV)	(0.5291-
	G	814 (0.68)	284 (0.57)	825 (0.57)	<0.0001[b]		0.7282)
					0.833[c]	0.079	
						(C)	0.9729[c]
							(0.7915-
							1.196)
	Genotype	(n = 599)	(n = 247)	(n = 726)			
	AA	0 (0.00)	0 (0.00)	0 (0.00)		<0.00	
	AG	0 (0.00)	0 (0.00)	0 (0.00)	1.000	01	1.000
rs1534284-	GC	599 (1.00)	247 (1.00)	726 (1.00)	(AV, SV)	(AV,	(0.801-
rs1534283	Allele					SV)	1.235)
(AA/GC;	G	599 (0.50)	247 (0.50)	726 (0.50)	1.000		(AV, SV)
Arg4Gln)	C	599 (0.50)	247 (0.50)	726 (0.50)	(AV, SV)	<0.00	
						01	
						(C)	

'n' represents number of Patients/ Controls,
HWE refers to Hardy-Weinberg Equilibrium,
AV refers to Active Vitiligo, SV refers to Stable Vitiligo and C refers to controls,
[a]Active Vitiligo vs. Stable Vitiligo,
[b]Active Vitiligo vs. Controls,
[c]Stable Vitiligo vs. Controls,
Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

6.3.4 Relative gene expression of *MYG1* in patients with vitiligo and controls

6.3.4 Relative gene expression of *MYG1* in patients with vitiligo and controls

Comparison of the findings showed significant increase in expression of *MYG1* transcripts in 166 vitiligo patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p<0.0001) (Figure 3A). Moreover, generalized vitiligo patients showed significant higher expression of *MYG1* transcripts as compared to localized vitiligo patients (p=0.001) (Figure 3A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.447 fold change in the expression of *MYG1* transcript in patients as compared to controls (Figure 4A).

6.3.5 Correlation of *MYG1* transcripts with the *MYG1* -119C/G genotypes

Further, the expression levels of *MYG1* were analyzed with respect to -119C/G genotypes (Figure 3B). Interestingly, *MYG1* expression was significantly increased in patients with susceptible GG genotypes as compared to controls (p=0.008). Also, patients with genotypes CG showed increased *MYG1* transcripts as compared to controls (p=0.025); however, no significant difference was observed in *MYG1* expression in patients as compared to controls with CC genotypes (p=0.078).

6.3.6 Effect of *MYG1* expression on disease progression

In addition, we also checked the effect of *MYG1* expression on progression of the disease i.e. active and stable cases (Figure 3C). Interestingly, active vitiligo patients showed significant increase in expression of *MYG1* transcripts as compared to the patients with stable vitiligo (p=0.0002) suggesting the involvement of *MYG1* in disease progression. Moreover, the susceptibility of the disease was also checked based on the gender differences and we found that female patients with vitiligo showed significantly higher *MYG1* expression as compared to male patients (p=0.008) (Figure 3D).

When *MYG1*expression was monitored in different age groups of patients, patients with the age group 1-20 yrs showed significantly increased expression of *MYG1* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.037,

p=0.008 and p=0.029 respectively) suggestive of the importance of *MYG*1 in early onset of the disease (Figure 4B).

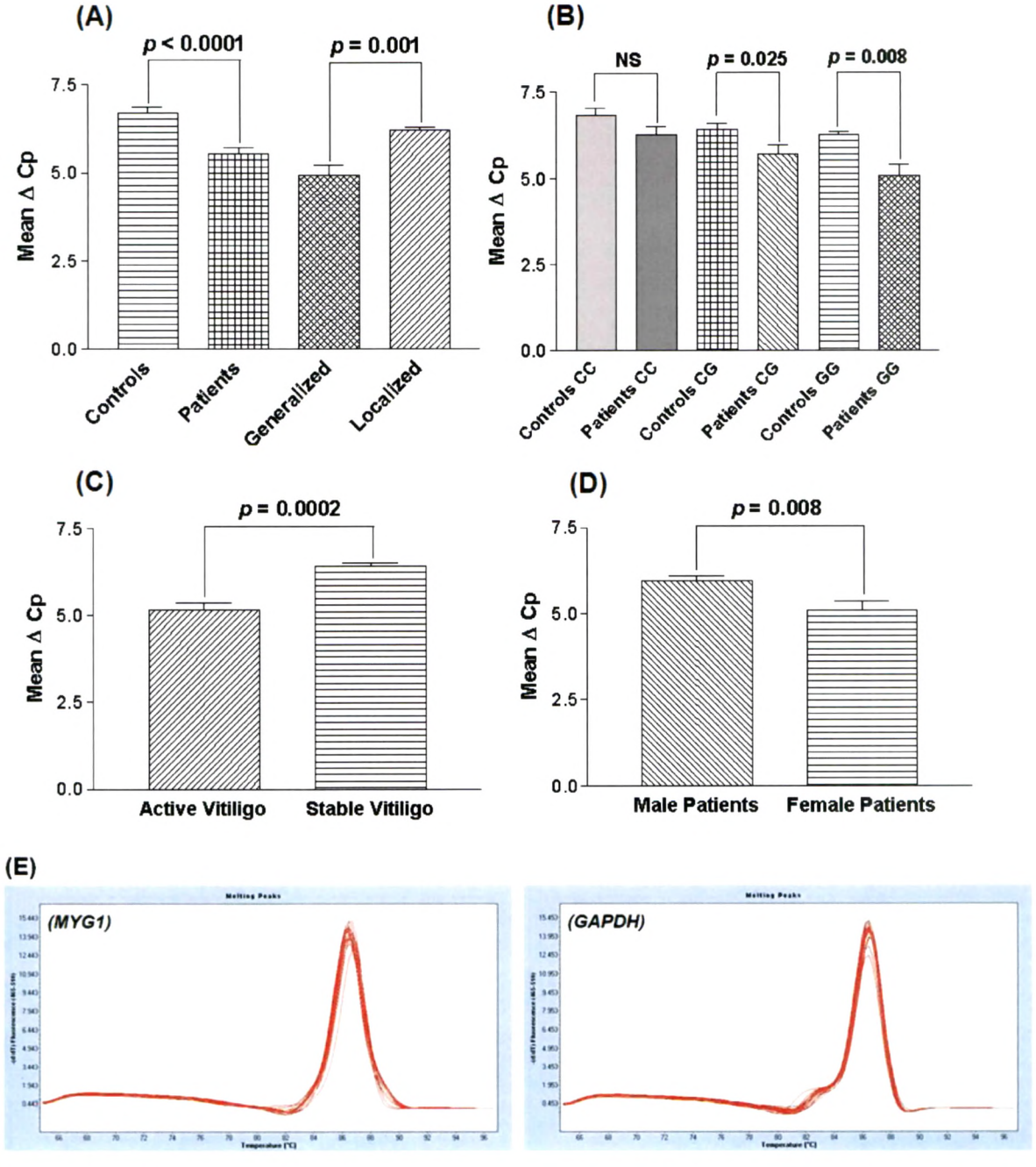

Figure 3. Relative gene expression of *MYG1* in controls and vitiligo patients:

(A) Expression of *MYG1* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp.

(B) Expression of *MYG1* transcripts with respect to *MYG1* promoter -119C/G in 166 vitiligo patients and 175 controls, as suggested by Mean ΔCp.

(C) Expression of *MYG1* transcripts with respect to activity of the disease in 121 patients with active vitiligo and 45 patients with stable vitiligo, as suggested by Mean ΔCp.

(D) Expression of *MYG1* transcripts with respect to gender differences in 87 male patients and 79 female patients with vitiligo, as suggested by Mean ΔCp.

(E) Melt curve analysis of *MYG1* and *GAPDH* showing specific amplification.

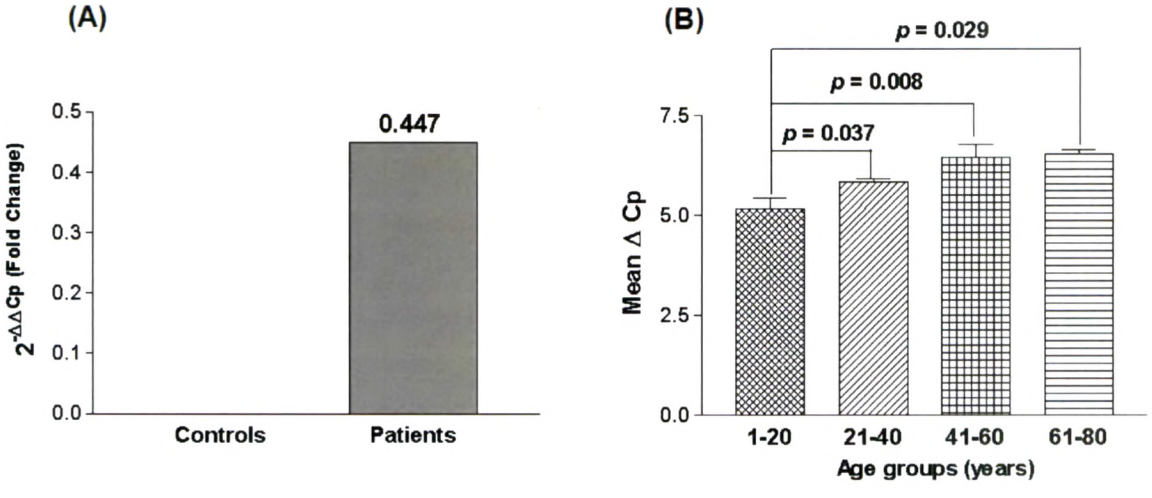

Figure 4. *MYG1* fold change and expression in different age groups of vitiligo:

(A) Expression fold change of *MYG1* transcripts in 166 vitiligo patients against 175 controls showed 0.447 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(B) Expression of *MYG1* transcripts with respect to different age groups in 166 vitiligo patients, as suggested by Mean ΔCp.

6.4 DISCUSSION

Vitiligo cannot be explained by simple Mendelian genetics, however it is characterized by incomplete penetrance, multiple susceptibility loci and genetic heterogeneity (Zhang *et al.*, 2005). It is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Nordlund, 1997; Kim *et al.*, 1998). Previous study suggests that 22% of Gujarat vitiligo patients exhibit positive family history and 14% patients have at least one first-degree relative affected (Shajil *et al.*, 2006b). Also, in the present study 13.71% of vitiligo patients have one or more first degree relative affected (Table 1) suggesting the involvement of genetic factors in vitiligo pathogenesis.

Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo since it shows frequent association with other autoimmune disorders and patients with the disease show autoantibodies to melanosomal proteins in the serum (Ochi and DeGroot, 1969; Baharav *et al.*, 1996; Kemp *et al.*, 1997). Moreover, autoreactive cytotoxic T lymphocytes (CTLs), which specifically recognize melanocyte differentiation antigens, have been detected in both the peripheral blood and perilesional skin of individuals with vitiligo (Lang *et al.*, 2001; Ogg *et al.*, 1998; Palermo *et al.*, 2001). About 25-30% of generalized vitiligo patients manifest at least one other autoimmune disease; particularly Graves' disease, autoimmune hypothyroidism, rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, Addison's disease, and systemic lupus erythematosus (Alkhateeb *et al.*, 2003; Laberge *et al.*, 2005). The destruction of melanocytes due to an autoimmune response in vitiligo can be either through cellular and/or humoral immune response (Shajil *et al.*, 2006a; Kemp *et al.*, 2001). Recently, Singh *et al.* (2012) have shown positive association of HLA-A*33:01, HLA-B*44:03, and HLA-DRB1*07:01 with vitiligo patients from North India and Gujarat suggesting an autoimmune link of vitiligo in these cohorts.

MYG1 gene was initially found to be highly expressed in freely proliferating mouse melanocytes compared with melanoma cells (Smicun, 2000). *MYG1* is predominantly expressed in the ectoderm derived tissues, including epidermis (Philips *et al.*, 2009)

indicating that MYG1 is involved in the development of skin, therefore MYG1 may have crucial role in skin diseases. According to Su *et al.,* (2004) the *MYG1* transcript levels in human skin is under average, compared with other human tissues. However, previous studies indicated that *MYG*1 is upregulated in the skin of patients with vitiligo and atopic eczema (Kingo *et al.*, 2006; Sääf et al., 2008). In the present study, we show that systemic expression of *MYG*1 transcript is elevated in vitiligo patients as compared to controls (Figure 3A). The increased expression of *MYG*1 transcript in generalized vitiligo patients as compared to patients with localized vitiligo (Figure 3A) suggests the crucial role of MYG1 in precipitation of generalized vitiligo. Moreover, the patients with active vitiligo had elevated levels of *MYG*1 transcript as compared to patients with stable vitiligo (Figure 3C) implicating its predominant role in progression of the disease, and our results are in line with the previous findings (Philips *et al.*, 2010; Sääf *et al.*, 2008). In addition, we found elevated *MYG1* transcript levels in the 1-20 yrs of age group of patients (Figure 4B) suggesting its involvement in early phase of the disease.

The gender based analysis had clearly showed the susceptibility of the disease towards female patients as they demonstrated elevated levels of *MYG1* transcript and an early age of onset of vitiligo as compared to male patients (Figure 3D). There are reports suggesting that vitiligo occurs twice as often in females than in males (Westerhof and d'Ischia, 2007). It seems estrogen may be involved in the depigmentation process of vitiligo, as the initiation/progression of the disease is observed at pregnancy, postpartum, in the menopause or after the use of oral contraceptives/hormonal substitution (Schallreuter *et al.*, 2006). Cheng *et al.,* (2006) identified *MYG1* as a putative estrogen receptor alpha target gene; therefore, it is possible that MYG1 can regulate hormonal balance directly through estrogen receptor.

Philips *et al.,* (2010) showed that *MYG1* mRNA levels in skin samples of healthy controls correlate with *MYG1* promoter polymorphism -119C/G, and subjects with homozygous -119G allele have significantly higher *MYG1* transcript levels than subjects with homozygous -119C allele. The study also confirmed higher activity of -119G allele by using *in vitro* luciferase reporter assay and showed alteration of p300 (E1A-associated 300 kDa protein) binding site is a potential reason for the *MYG1*

-119G promoter allele to be more active. The -119G allele was on average more than 2.5 fold more active regardless of the length of the promoter fragment (Philips *et al.*, 2010).

In the present study, we found that -119G allele was more frequent in patients as compared to controls (Table 3) and our results are in line with those of Philips *et. al.* (2010). Further, the genotype-phenotype analysis of -119C/G SNP revealed that patients with GG and CG genotype had increased *MYG1* expression as compared to controls (Figure 3B) suggesting that -119G allele is transcriptionally more active and hence elevated levels of *MYG1* transcripts are seen in patients. Thus, vitiligo patients with -119G promoter allele seem to have genetic susceptibility to vitiligo. However, Philips *et al.*, (2010) showed that the minor allele -119G was related to higher *MYG1* transcript levels only in control group, but not in vitiligo group. Interestingly, in the present study -119G allele was prevalent in active vitiligo patients as compared to patients with stable vitiligo and control group (Table 6) which suggests the crucial role of -119G allele in susceptibility as well as progression of the disease. In addition, we found that patients with *MYG1*-119GG genotype had an early onset of the disease as compared to CG and CC genotypes (Figure 2A) suggesting the participation of MYG1 in early events of the disease. Furthermore, gender based analysis revealed sex-biasness in distribution of -119G allele which is prevalent in female patients as compared to male patients (Table 5) supporting the fact that females are more susceptible for acquring autoimmune diseases like vitiligo.

Genetic association analysis revealed that rs1534284/rs1534283 double-polymorphism was likewise prevalently monogenic in Gujarat population with only *MYG1* 4Arg allele being present (Table 3). Previously, this polymorphism was also found to be monogenic in Estonian population (Philips *et al.*, 2010). According to currently available data, there can be either arginine or glutamine at the fourth position of MYG1 in humans. Philips *et al.*, (2010) showed that only positively charged arginine at the fourth position enables mitochondrial entry of MYG1 which has crucial functions inside mitochondria.

The acidic glutamine disturbs a common property of mitochondrial targeting sequence to form an amphiphilic helical structure that is essential for the effective transport of a mitochondrial protein (Shimoda-Matsubayashi, 1996). According to HapMap database heterozygosity for *MYG1* Arg4Gln polymorphism (rs1534284) has been currently detected only in YRI (Yoruba in Ibadan) population from Nigeria. Among 57 subjects from YRI population who were genotyped for HapMap project, 20 subjects (35.1%) were heterozygous for *MYG1* Arg4Gln polymorphism but there are no subjects who are homozygous for *MYG1* 4Gln allele. According to currently available data, *MYG1* 4Gln allele that disturbs mitochondrial entry of MYG1 has never been detected in the homozygous state. Hence, it was proposed that persons with *MYG1* 4Gln variant do not survive or have a health condition that keeps them out from the study groups (Philips *et al.*, 2010).

The genetic association analysis and expression studies suggest that *MYG1* is one of the genes that in interaction with other genetic and environmental factors is responsible for the development of vitiligo. However, *MYG1* expression in the skin seems to be not specific to melanocytes as *MYG1* expression in cultured melanocytes is lower than expression in full skin biopsy and comparable or equal with *MYG1* expression in cultured fibroblasts derived from the same skin (Philips *et al.*, 2010). Therefore, the precise function of MYG1 in the development of vitiligo is still unclear but the up-regulation of several immune system-related genes after *MYG1* siRNA knockdown in cell culture (Philips *et al.*, 2009) suggests that MYG1 can act as a mediator in the immune processes that are disturbed in vitiligo patients.

Philips *et. al.*, (2009) showed that siRNA mediated knockdown of *MYG1* mRNA resulted in the altered levels of transcripts encoding transcription factors involved in development, tissue patterning and growth as well as immune-related processes. The study (Philips *et al.*, 2009) found that immune reaction-related genes were consistently upregulated after *MYG1* knockdown i.e. *GBP1* (guanylate-binding protein 1), *GBP2* (guanylate binding protein 2), *CD59* (complement regulatory protein) and *UL16*-binding protein 2. Previously, *MYG1* mRNA expression was found to be decreased in $CD8^+$ cytotoxic T-lymphocytes after interleukin-12-induced activation in a mouse mammary carcinoma model (Cao *et al.*, 2004) and in response

to *in vitro* treatment of peripheral blood cells with IFN-α (Taylor *et al.,* 2004). Additionally, upregulation of *MYG1* mRNA has been reported in a methylcholanthrene-induced sarcoma tumour model following treatment with the anti-inflammatory COX (cyclo-oxygenase)-inhibitor indomethacin (Axelsson *et al.,* 2007). These reports support the involvement of MYG1 in immune regulatory functions and hence it becomes pertinent to investigate *MYG1* gene especially in case of generalized vitiligo which is considered to be of autoimmune origin.

In conclusion, our results confirm that -119C/G polymorphism influences *MYG1* transcript levels and minor -119G allele is the risk-allele for the development of vitiligo. The study also emphasizes the influence of MYG1 on the disease progression, onset of the disease and gender biasness for developing vitiligo. More detailed studies will be needed to confirm the genetic association of *MYG1* promoter and structural polymorphisms and to understand the precise function of MYG1 in vitiligo pathogenesis.

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CHAPTER VII

NACHT LEUCINE-RICH REPEAT PROTEIN 1 (*NALP1*) GENE PROMOTER AND STRUCTURAL POLYMORPHISMS AND THEIR GENOTYPE-PHENOTYPE CORRELATION WITH GENERALIZED VITILIGO SUSCEPTIBILITY

7.1 INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder in which progressive, patchy loss of pigmentation from skin, overlying hair, and oral mucosa results from autoimmune loss of melanocytes from the involved areas (Nordlund *et al.*, 2006). It appears to be a polygenic, multifactorial disorder involving both multiple susceptibility genes (Majumder *et al.*, 1993; Nath *et al.*, 1994; Sun *et al.*, 2006), perhaps in different combinations in different populations, and as-yet unknown environmental triggers. Epidemiological studies have shown frequent family clustering of vitiligo cases, with elevated risk of vitiligo in first-degree relatives and high concordance in monozygotic twins (Alkhateeb *et al.*, 2003; Sun *et al.*, 2006). Approximately one in four vitiligo patients manifest another autoimmune disorder, principally autoimmune thyroid disease, rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, Addison's disease, and systemic lupus erythematosus (Alkhateeb *et al.*, 2003; Laberge *et al.*, 2005). Furthermore, these autoimmune diseases also occur at elevated frequency among vitiligo patient's close relatives, suggesting that increased risk of vitiligo and other autoimmune disorders in these families has a genetic basis.

A number of genes have been implicated in the pathogenesis of generalized vitiligo on the basis of genetic linkage and association studies (reviewed in Spritz, 2008). Recent genome-wide association studies (GWAS) revealed a highly significant association of familial cases of generalized vitiligo with polymorphic variants of the gene encoding *NALP1* - a key regulator of the innate immune system (also known as *NLRP1*; Ting *et al.*, 2008) (Jin *et al.*, 2007a).

NALP1 is a critical component of the surveillance system that detects bacterial and viral infections thus acts as a primary regulator of the innate immune system. *NALP1* is mainly expressed in Langerhans cells and T cells (Kummer *et al.*, 2007). NALP1 is thought to recognize pathogen-associated molecular patterns, which would trigger the assembly of the NALP1 inflammosome and consequently stimulate inflammatory response and apoptotic pathways (Church *et al.*, 2008).

The *NALP1* gene encodes NLRP1, a leucine-rich repeat protein 1, a member of the nucleotide oligomerization domains-like receptors (NLRs) family. The NLRs are a group of cytoplasmic pattern recognition receptors, which nonspecifically recognize microbial products, such as lipopolysaccharide, thereby stimulating innate immunity. NALP1 contains five domains, a protein-protein interaction N-terminal pyrin domain followed by centrally located NACHT (NAIP CIIA HET-E and TP1 family proteins) domain responsible for oligomerization and activation of the NLRs, a domain comprised of five tandem leucine-rich repeat (LRR) domains that is speculated to bind microbial ligands, a FIIND domain, and a finally a C-terminal caspase recruitment (CARD) domain (Martinon and Tschopp, 2005). Activated NALP1 interacts directly with caspase-1 and caspase-5, forming a multiprotein complex known as the inflammasome, which promotes the processing and maturation of cytokines such as IL1, IL18, and IL33 (Tschopp *et al.*, 2003; Faustin *et al.*, 2007). Through its multiple domain structure, NALP1 plays a crucial role in the assembly of the apoptosome, a complex composed of NALP1, caspase-2, and caspase-9. High levels of expression in immune cells, particularly T cells and Langerhans' cells, emphasize the role of NLRP1 in regulation of the immune system (Ting and Davis, 2005).

Earlier studies showed variants of *NALP1* to be associated with vitiligo in Caucasian patients from the USA, UK, Romania and Jordanian populations (Jin *et al.*, 2007a,b,; Alkhateeb and Qarqaz, 2010). Other studies have also demonstrated that polymorphisms in *NLRP*1 are associated with autoimmune conditions like Addison's disease, and type 1 diabetes (Magitta *et al.*, 2009; Zurawek *et al.*, 2010).

The objectives of this study were:

i.) To measure and compare *NALP1* transcript levels in patients with vitiligo and in unaffected controls.

ii.) To determine whether *NALP1* promoter (A/G; rs2670660), structural (A/T; rs12150220) and intronic (T/C rs6502867) polymorphisms are associated with vitiligo susceptibility and modulate *NALP1* transcript levels in these groups.

iii.) To correlate *NALP1* polymorphisms/levels with the onset and progression of disease.

7.2 MATERIALS AND METHODS

7.2.1 Study Subjects:

The study group included 537 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 237 males and 300 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. A total of 645 ethnically sex-matched unaffected individuals (295 males and 350 females) were included in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of generalized vitiligo patients and unaffected controls.

	Generalized Vitiligo (GV) Patients	**Controls**
	(n = 537)	(n = 645)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	237 (44.13%)	295 (45.74%)
Female	300 (55.87%)	350 (54.26%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Type of vitiligo		
Active vitiligo	392 (73.00%)	NA
Stable vitiligo	145 (27.00%)	NA
Family history	71 (13.22%)	NA

7.2.2 Genomic DNA Preparation:

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

7.2.3 Genotyping of *NALP1* A/G promoter (rs2670660) and T/C intronic (rs6502867) polymorphisms:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype A/G (rs2670660) and T/C (rs6502867) polymorphisms of *NALP1* gene (Figure 1A & B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes (New England Biolabs, Beverly, MA) *Apo*I and *Hpy*8I were used for digesting amplicons of A/G (rs2670660) and T/C (rs6502867) of *NALP1* gene respectively (Table 2). 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 50 bp or 100 bp DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the

samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

Table 2. Primers and restriction enzymes used for *NALP1* A/G promoter (rs2670660) and T/C intronic (rs6502867) SNPs genotyping and gene expression analyses.

Gene/SNP Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs2670660)				*Apo*I
NALP1 A/G F	TGTTTATCCCAGGGCTTCTTGT	53	227	(179 &
NALP1 A/G R	CAGTGTTCTGTGGAAATGAAAGAG			48 bp)
(rs6502867)				*Hpy*8I
NALP1 T/C F	TGGGGGTTTGGGTTCTTAG	56	216	(125 &
NALP1 T/C R	TTTCTCATGATAGTTTGGGTGTG			91 bp)
NALP1 expression F	CGCCGCTGGAGAGAAATCTC	65	232	-
NALP1 expression R	CTCTCTCTTTCTCTGATTTCTGTGT			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expression R	CAAATGAGCCCCAGCCTTCT			

7.2.4 Genotyping of *NALP1* A/T structural (rs12150220; Leu155His) polymorphism:

The genotyping of A/T (rs12150220) SNP of *NALP1* was carried out by dual color hydrolysis probes (FAM and VIC) using LightCycler® 480 Real-Time PCR protocol with background corrected end point fluoroscence analysis using TaqMan SNP genotyping assay (Assay ID: C_1600653_10; Life Technologies Corp., California, USA). Real-time PCR was performed in 10 µl volume using LightCycler® 480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) following the

manufacturer's instructions. A no-template control (NTC) was used with the SNP genotyping assay.

7.2.5 Determination of *NALP1* and *GAPDH* mRNA expression:

7.2.5.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

7.2.5.2 Real-time PCR:

The expression of *NALP1* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (94°C for 15 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 4I). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

7.2.6 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all three polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis. The distribution of the genotypes and allele frequencies of *NALP1* polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.017 were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated.
Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi *et al.*, 2005). The linkage disequilibrium (LD) coefficients D' = D/Dmax and r^2-values for the pair of the most common alleles at each site were estimated using the Haploview programe version 4.1 (Barrett *et al.*, 2005). Age of onset analysis and relative expression of *NALP1* in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al.*, 2007).

7.3 RESULTS

7.3.1 Analysis of association between *NALP1* (A/G; rs2670660) promoter polymorphism and susceptibility to vitiligo:

PCR-RFLP for *NALP1* A/G promoter polymorphism yielded a 227 bp undigested product corresponding to 'G' allele and 179 bp and 48 bp digested products corresponding to 'A' allele. The three genotypes identified by 2.5% agarose gel electrophoresis were: AA homozygous, AG heterozygous and GG homozygous for A/G polymorphism of *NALP1* gene (Figure 1A).

The genotype and allele frequencies of the A/G polymorphism in 537 vitiligo patients and 645 controls are summarized in Table 3. The *NALP1* A/G promoter

polymorphism was found to be in significant association with vitiligo patients (p<0.0001) when genotypes were compared using chi-squared test-3x2 contingency table (Table 3). In particular, the minor allele (G) of the *NALP1* A/G polymorphism was more frequent in the vitiligo group compared to the control group (51.0% versus 42.0%, p<0.0001) (Table 3). Both control and patient groups were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.062 and p=0.135 respectively) (Table 3).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the minor 'G' allele of *NALP1* A/G promoter polymorphism occurred prevalently in the group of patients with active vitiligo (56.0% versus 42.0%, p<0.0001) compared to the control group (Table 4). However, there was no statistically significant difference in the distribution of the 'G' allele between patients with stable vitiligo and control group (p=0.235) (Table 4). Interestingly, the 'G' allele was more prevalent in patients with active vitiligo as compared to stable vitiligo (56.0% versus 38.0%, p<0.0001) suggesting the important role of 'G' allele in progression of the disease. In addition, gender based analysis of *NALP1* A/G polymorphism suggested significant association of minor 'G' allele, consistent with a susceptibility effect with female vitligo patients as compared to male patients (55.0% versus 47.0%, p=0.007, OR: 1.399, 95% CI: 1.099-1.782 (Table 5). This study has 86.71% statistical power for the effect size 0.08 to detect the association of investigated polymorphisms at p<0.05 in generalized vitiligo patients and control population.

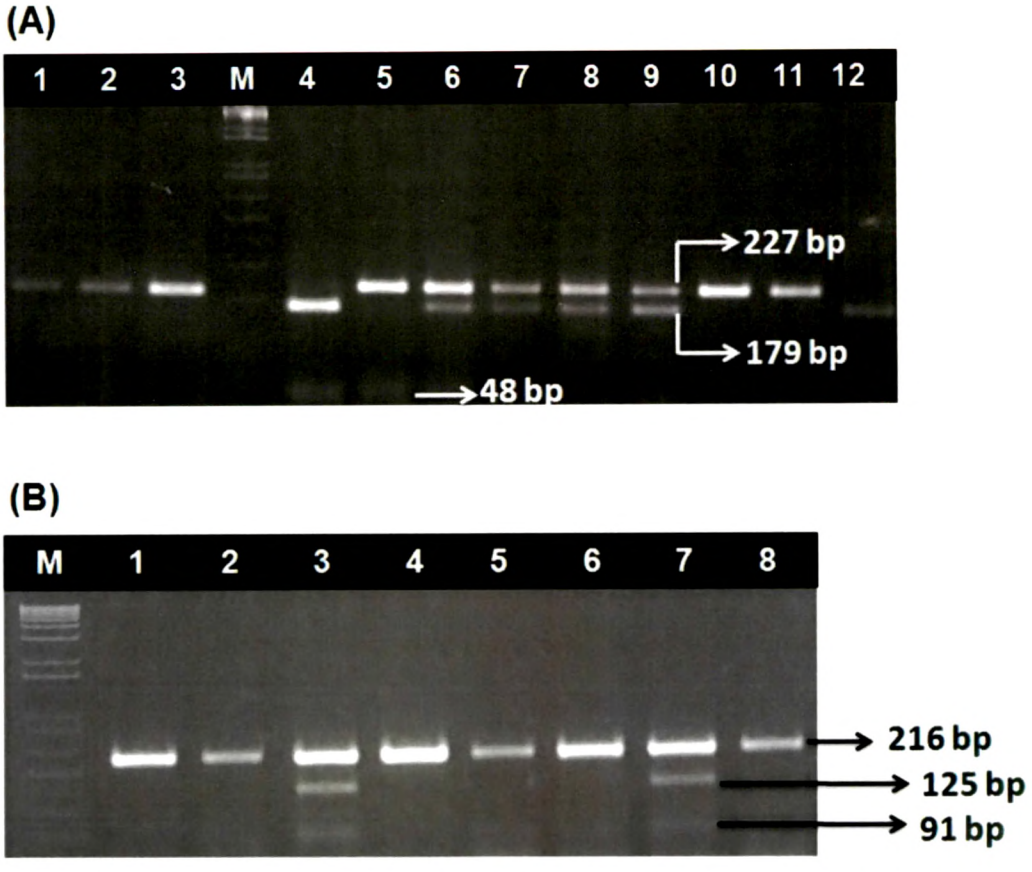


Figure 1. PCR-RFLP analysis of *NALP1* A/G (rs2670660) and C/T (rs6502867) polymorphisms:

(A) PCR-RFLP analysis of *NALP1* A/G (rs2670660) promoter polymorphism on 2.5 % agarose gel electrophoresis: lanes: 4 &12 show homozygous (AA) genotypes; lanes: 6, 7, 8, & 9 show heterozygous (AG) genotypes; lanes: 1, 2, 3, 5, 10 & 11 show homozygous (GG) genotypes; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *NALP1* C/T (rs6502867) intron polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 2, 4, 5, 6 & 8 show homozygous (CC) genotypes; lanes: 3, & 7 show heterozygous (CT) genotypes; lane M shows 50 bp DNA ladder.

7.3.2 Analysis of association between *NALP1* (T/C; rs6502867) polymorphism and susceptibility to vitiligo:

PCR-RFLP for *NALP1* T/C intron polymorphism yielded a 216 bp undigested product corresponding to 'C' allele and 125 bp and 91 bp digested products corresponding to 'T' allele. The three genotypes identified by 2.5% agarose gel electrophoresis were: TT homozygous, TC heterozygous and CC homozygous for T/C polymorphism of *NALP1* gene (Figure 1B).

The genotype and allele frequencies of the T/C polymorphism in 537 vitiligo patients and 645 controls are summarized in Table 3. The *NALP1* T/C polymorphism was found to be in significant association with vitiligo patients ($p<0.0001$) when genotypes were compared using chi-squared test-3x2 contingency table (Table 3). In particular, the minor allele 'C' of the *NALP1* T/C polymorphism was more frequent in the vitiligo group compared to the control group (41.0% versus 31.0%, $p<0.0001$) (Table 3). Both control and patient groups were found to be in Hardy-Weinberg equilibrium for this polymorphism ($p=0.096$ and $p=0.071$ respectively) (Table 3).
Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the minor 'C' allele of *NALP1* T/C polymorphism occurred prevalently in the group of patients with active vitiligo (44.0% versus 31.0%, $p<0.0001$) compared to the control group (Table 4). However, there was no statistically significant difference in the distribution of the 'C' allele between patients with stable vitiligo and control group (p=0.676) (Table 4). Interestingly, the 'C' allele was more prevalent in patients with active vitiligo as compared to stable vitiligo (44.0% versus 33.0%, p=0.001) suggesting the important role of 'C' allele in progression of the disease. In addition, gender based analysis of *NALP1* T/C polymorphism suggested significant association of minor 'C' allele, consistent with a susceptibility effect with female vitligo patients as compared to male patients (45.0% versus 35.0%, p=0.001, OR 1.553, 95% CI 1.212-1.990 (Table 5). This study has 86.71% statistical power for the effect size 0.08 to detect the association of investigated polymorphisms at $p<0.05$ in generalized vitiligo patients and control population.

7.3.3 Analysis of association between *NALP1* (A/T; rs12150220) polymorphism and susceptibility to vitiligo:

Real time PCR based TaqMan SNP genotyping method was carried out by dual color hydrolysis probes labbled with FAM (for 'T' allele) and VIC (for 'A' allele) fluorophores for *NALP1* A/T (leu155his) polymorphism which yielded the three genotypes (AA homozygous, AT heterozygous and TT homozygous) as identified by scattered plot using background corrected end point fluoroscence analysis (Figure 2). The genotype and allele frequencies of the *NALP1* A/T (rs12150220) polymorphism in 537 vitiligo patients and 645 controls are summarized in Table 3. The *NALP1* A/T polymorphism was not found to be in significant association with vitiligo patients as suggested by the genotype and allele frequencies for the polymorphism (p=0.074; p=0.012) (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.052 and p=0.728 respectively) (Table 3).

Analysis based on the stage of progression of vitiligo revealed that there was no significant difference in the distribution of minor 'T' allele of *NALP1* A/T polymorphism between patients with active vitiligo and control group (p=0.0.063) (Table 4). Also, there was no statistically significant difference in the distribution of the 'T' allele between both the groups of patients with active and stable vitiligo and control group (p=1.00; p=0.215) (Table 4). No significant difference was obsereved in 'T' allele frequency between male and female patients (p=0.527) (Table 5).This study has 86.71% statistical power for the effect size 0.08 to detect the association of *NALP1* A/T (leu155his) polymorphism at $p<0.05$ in generalized vitiligo patients and control population.

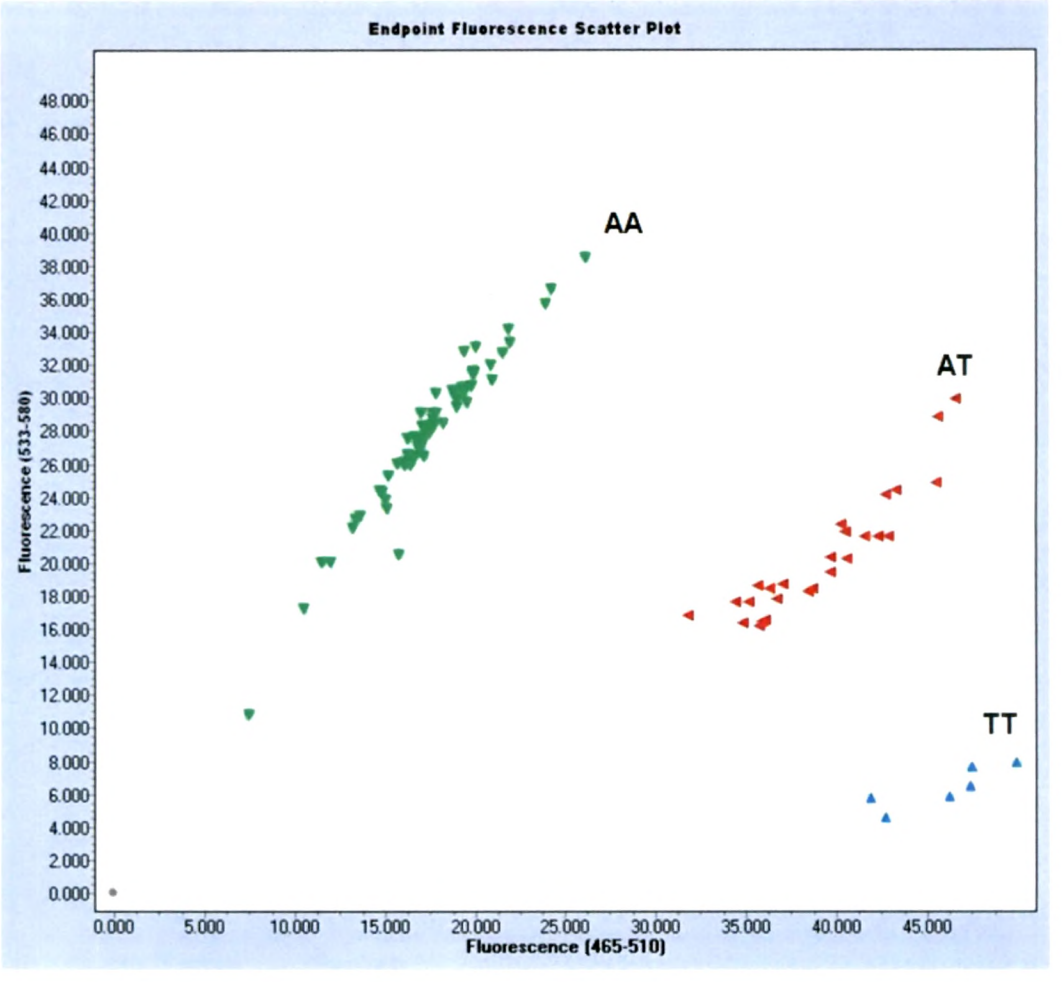


Figure 2. TaqMan end point fluoroscence analysis for *NALP1* A/T (rs12150220) using dual color hydrolysis probes (FAM and VIC) by LightCycler® 480Real-Time PCR protocol. The three genotypes identified as: AA, AT and TT, based on fluorescence with Channel 465-510 (FAM for 'T' allele) and Channel 536-580 (VIC for 'A' allele). A no-template control (NTC) was used with the SNP genotyping assay.

Table 3. Association studies for *NALP1* gene, A/G (rs2670660) promoter, T/C (rs6502867) intron and A/T (rs12150220) polymorphisms in vitiligo patients from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 537)	(n = 645)			
	AA	136 (0.25)	229 (0.36)		0.135	
rs2670660	AG	251 (0.47)	291 (0.45)	<0.0001[a]	(P)	
(A/G)	GG	150 (0.28)	125 (0.19)		0.062	
	Allele				(C)	0.6856
	A	523 (0.49)	749 (0.58)	<0.0001[b]		(0.5825-
	G	551 (0.51)	541 (0.42)			0.8069)
	Genotype	(n = 537)	(n = 645)			
	TT	199 (0.37)	312 (0.48)		0.071	
rs6502867	TC	239 (0.44)	260 (0.40)	<0.0001[a]	(P)	
(T/C)	CC	99 (0.18)	73 (0.12)		0.096	
	Allele				(C)	0.6695
	T	637 (0.59)	884 (0.69)	<0.0001[b]		(0.5652-
	C	437 (0.41)	406 (0.31)			0.7929)
	Genotype	(n= 537)	(n = 645)			
	AA	298 (0.55)	400(0.62)		0.728	
rs12150220	AT	202 (0.38)	205 (0.32)	0.074[a]	(P)	
(A/T)	TT	37 (0.07)	40 (0.06)		0.052	
	Allele				(C)	0.7813
	A	788 (0.73)	1005(0.78)	0.012[b]		(0.6469-
	T	286 (0.27)	285 (0.22)			0.9436)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.017$ due to Bonferroni's correction.

Table 4. Association studies for *NALP1* gene, A/G (rs2670660) promoter, T/C (rs6502867) intron and A/T (rs12150220) polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 392)	(n = 145)	(n = 645)			0.4753 [a]
	AA	77 (0.20)	59 (0.40)	229(0.36)	<0.0001[a]	0.686	(0.3606-
rs2670660	AG	189 (0.48)	62 (0.43)	291(0.45)	<0.0001[b]	(AV)	0.6264)
(A/G)	GG	126 (0.32)	24 (0.17)	125(0.19)	0.467[c]		0.5618 [b]
						0.268	(0.4696-
	Allele					(SV)	0.6721)
	A	343 (0.44)	180 (0.62)	749(0.58)	<0.0001 [a]		1.182[c]
	G	441 (0.56)	110 (0.38)	541(0.42)	<0.0001[b]	0.062	(0.9097-
					0.235[c]	(C)	1.536)
	Genotype	(n = 392)	(n = 145)	(n = 645)			0.6296 [a]
	TT	129 (0.33)	70 (0.48)	312(0.48)	0.004[a]	0.366	(0.4744-
	TC	184 (0.47)	55 (0.38)	260(0.40)	<0.0001[b]	(AV)	0.8356)
rs6502867	CC	79 (0.20)	20 (0.14)	73 (0.12)	0.676[c]		1.685 [b]
	Allele					0.094	(1.402-
(T/C)	T	442 (0.56)	195 (0.67)	884(0.69)	0.001 [a]	(SV)	2.024)
	C	342 (0.44)	95 (0.33)	406(0.31)	<0.0001[b]		1.061 [c]
					0.676[c]	0.096	(0.8081-
						(C)	1.392)
	Genotype	(n = 392)	(n = 145)	(n = 645)			0.9871 [a]
	AA	216 (0.55)	82 (0.57)	400 (0.62)	0.843[a]	0.995	(0.7248-
rs12150220	AT	150 (0.38)	52 (0.36)	205 (0.32)	0.081[b]	(AV)	1.344)
(A/T)	TT	26 (0.07)	11 (0.07)	40 (0.06)	0.464[c]		0.8171 [b]
	Allele					0.496	(0.6641-
	A	582 (0.74)	216 (0.74)	1005(0.78)	1.000 [a]	(SV)	1.005)
	T	202 (0.26)	74 (0.26)	285 (0.22)	0.063[b]		1.208 [c]
					0.215[c]	0.052	(0.8994-
						(C)	1.623)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

AV refers to Active Vitiligo, SV refers to Stable Vitiligo and C refers to controls,

[a]Active Vitiligo vs. Stable Vitiligo,

[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Values are significant at $p \leq 0.017$ due to Bonferroni's correction.

Table 5. Association studies for *NALP1* gene, A/G (rs2670660) promoter, T/C (rs6502867) intron and A/T (rs12150220) polymorphisms in male and female patients with vitiligo.

SNP	Genotype or allele	Male Patients (Freq.)	Female Patients (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 237)	(n = 300)			
	AA	74 (0.31)	62 (0.20)	0.017[a]	0.091	
rs2670660	AG	105 (0.44)	146 (0.50)		(M)	
(A/G)	GG	58 (0.25)	92 (0.30)			
	Allele				0.771	
	A	253 (0.53)	270 (0.45)	0.007[b]	(F)	1.399
	G	221 (0.47)	330 (0.55)			(1.099-1.782)
	Genotype	(n = 237)	(n = 300)			
	TT	105 (0.44)	94 (0.31)		0.220	
rs6502867	TC	99 (0.42)	140 (0.47)	0.003[a]	(M)	
(T/C)	CC	33 (0.14)	66 (0.22)			
	Allele				0.311	
	T	309 (0.65)	328 (0.55)	0.001[b]	(F)	1.553
	C	165 (0.35)	272 (0.45)			(1.212-1.990)
	Genotype	(n= 237)	(n = 300)			
	AA	136 (0.57)	162 (0.54)		0.586	
rs12150220	AT	85 (0.36)	117 (0.39)	0.729[a]	(M)	
(A/T; Leu155His)	TT	16 (0.07)	21 (0.07)			
	Allele				0.984	
	A	357 (0.75)	441 (0.74)	0.527[b]	(F)	1.100
	T	117 (0.25)	159 (0.26)			(0.8343-1.451)

'n' represents number of Patients,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(M) refers to Male Patients and (F) refers to Female Patients,

[a] Male Patients vs. Female Patients using chi-squared test with 3 × 2 contingency table,

[b]Male Patients vs. Female Patients using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.017$ due to Bonferroni's correction.

7.3.4 Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the three polymorphisms investigated in the *NALP1* gene were in low LD association [**A/G: T/C** (D' =0.082, r^2=0.003); **A/G: A/T** (D'= 0.206, r^2=0.020); **T/C: A/T** (D'= 0.089, r^2=0.006)]. A haplotype evaluation of the three polymorphic sites was performed and the estimated frequencies of the haplotypes differed significantly between vitiligo patients and controls (global *p*-value=1.54e-008) (Table 6).

Table 6. Distribution of haplotypes frequencies for *NALP1* gene, A/G (rs2670660) promoter, T/C (rs6502867) intron and A/T (rs12150220; leu155his) polymorphisms among generalized vitiligo patients and controls.

Haplotype (A/G (rs2670660), T/C (rs6502867) and A/T (rs12150220)	Generalized Vitiligo Patients (Freq. %) (n=966)	Controls (Freq. %) (n=1066)	*p* for Association	$p_{(global)}$	Odds ratio (95% CI)
A C A	142.63(0.148)	170.64(0.160)	0.444	1.54e-008	0.910 [0.715~1.159]
A C T	41.91(0.043)	56.25(0.053)	0.327		0.815 [0.541~1.228]
A T A	196.76(0.204)	312.78(0.293)	3.42e-006		0.617 [0.503~0.757]
A T T	73.70(0.076)	85.33(0.080)	0.759		0.950 [0.687~1.315]
G C A	100.25(0.104)	69.26(0.065)	0.002		1.668 [1.212~2.296]
G C T	92.22(0.095)	51.85(0.049)	3.94e-005		2.067 [1.453~2.939]
G T A	205.37(0.213)	235.32(0.221)	0.665		0.954 [0.772~1.179]
G T T	112.17(0.116)	84.57(0.079)	0.01		1.526 [1.134~2.054]

CI represents Confidence Interval,

(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

Interestingly, the frequency of susceptible haplotype 'GCT' containing the minor alleles of all the three polymorphisms were significantly higher in vitiligo patients as compared to controls (9.5% vs 4.9%; p=3.94e-005) and increased the risk of vitiligo by 2.0-fold [odds ratio (OR): 2.067; 95% confidence interval (CI): (1.453~2.939)] (Table 6). Also two other haplotypes: 'GCA' and 'GTT' were significantly increased in vitiligo patients as compared to controls (p=0.002, p=0.01). However, the wild type haplotype 'ATA' was more frequently observed in control group as compared to the patient group (20% vs 29.3%; p= 3.42e-006) (Table 6).

7.3.5 Effect of *NALP1* polymorphisms on age of onset of vitiligo:

When age of onset of the disease was correlated with the *NALP1* A/G (rs2670660) promoter genotypes, patients with susceptible GG genotypes showed early onset of the disease as compared to AA and AG genotypes (p<0.0001 and p=0.011 respectively) (Figure 3A). Moreover, patients with genotype AG showed early onset of the disease as compared to AA genotypes (p=0.003) (Figure 3A) suggesting the effect of the susceptible allele 'G' on the early onset of disease.

Analysis of age of onset with *NALP1* T/C (rs6502867) genotypes revealed that patients with susceptible CC genotypes showed early onset of the disease as compared to TT and TC genotypes (p<0.0001 and p=0.0003 respectively) (Figure 3B). Moreover, patients with genotype TC showed early onset of the disease as compared to TT genotypes (p=0.032) (Figure 3B) suggesting the effect of the susceptible allele 'C' on the early onset of disease.

However, patients with *NALP1* (rs12150220) AA genotypes did not show any significant difference for the age of onset of the disease as compared to TT (p=0.163) and AT genotypes (p=0.635) (Figure 3C). Also, patients with AT genotype did not show significant difference in age of onset of disease as compared to TT genotype (p=0.357) (Figure 3C).

Interestingly, when male and female vitiligo patients were analyzed for age of onset of the disease, female patients had significant early onset of the disease as compared to the male patients (p<0.0001) (Figure 3D).

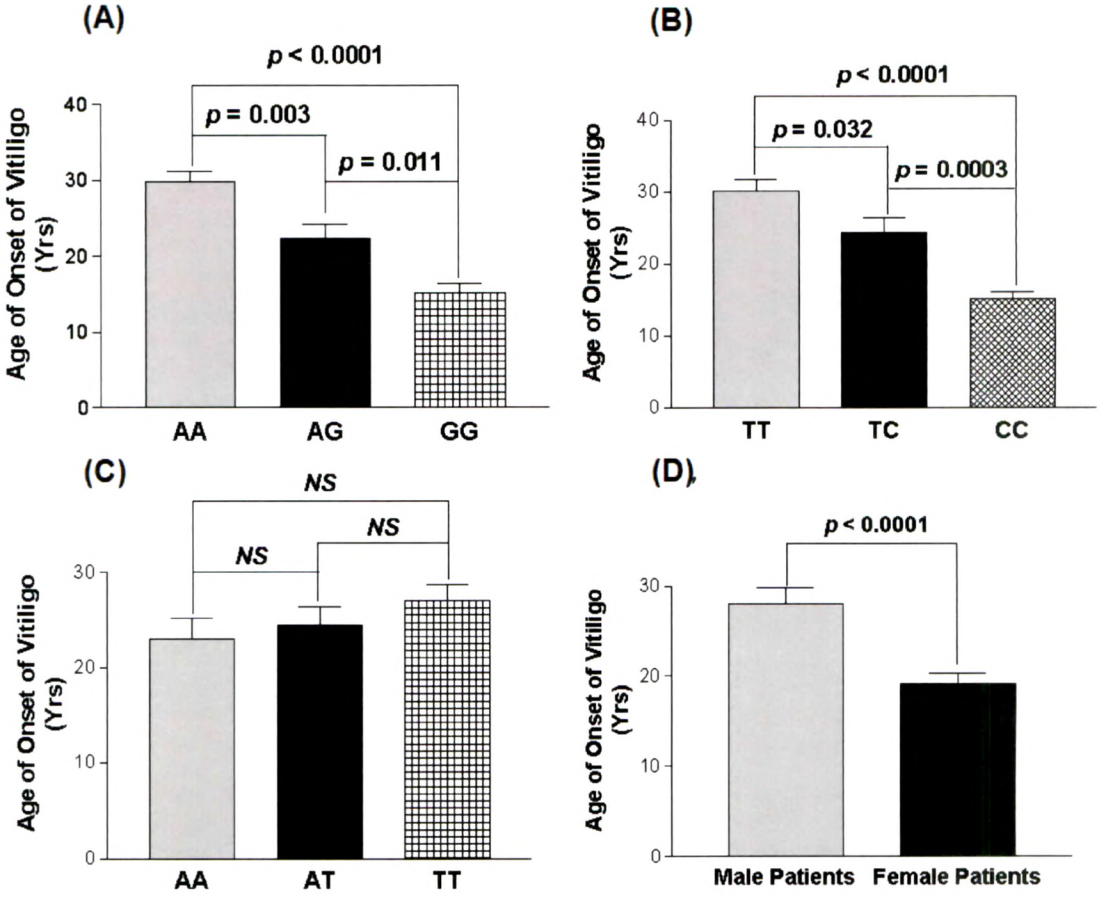


Figure 3. Age of onset of the disease in vitiligo patients:

(A) Comparison of age of onset of the disease (Years) with respect to *NALP1* A/G (rs2670660) polymorphism in 537 vitiligo patients. Vitiligo patients with GG genotype showed early age of onset of disease as compared to AA (Mean age of onset ± SEM: 15.13 ± 1.342 vs 29.77 ± 1.497; p<0.0001) and AG genotypes (Mean age of onset ± SEM: 15.13 ± 1.342 vs 22.42 ± 1.767; p=0.011). Patients with AG genotype showed early age of onset of disease as compared to AA genotype (Mean age of onset ± SEM: 22.42 ± 1.767 vs 29.77 ± 1.497; p=0.003).

(B) Comparison of age of onset of the disease (Years) with respect to *NALP1* T/C (rs6502867) polymorphism in 537 vitiligo patients. Vitiligo patients with CC genotype showed early age of onset of disease as compared to TT (Mean age of onset ± SEM: 15.13 ± 1.045 vs 30.13 ± 1.544; p<0.0001) and TC genotypes (Mean age of onset ± SEM: 15.13 ± 1.045 vs 24.40 ± 2.032; p=0.0003). Patients with TC genotype

showed early age of onset of disease as compared to TT genotype (Mean age of onset ± SEM: 24.40 ± 2.032 vs 30.13 ± 1.544; p=0.032).

(C) Comparison of age of onset of the disease (Years) with respect to *NALP1* A/T (rs12150220) polymorphism in 537 vitiligo patients. No significant diiference was observed in age of onset between Vitiligo patients with AA and TT genotypes (Mean age of onset ± SEM: 23.09 ± 2.097 vs 27.02 ± 1.874; p=0.163) and AT genotypes (Mean age of onset ± SEM: 23.09 ± 2.097 vs 24.48 ± 2.000; p=0.635). Also, patients with AT genotype did not show significant difference in age of onset of disease as compared to TT genotype (Mean age of onset ± SEM: 24.48 ± 2.000 vs 27.02 ± 1.874; p=0.357).

(D) Comparison of age of onset of the disease (Years) with respect to gender differences in 237 male patients and 300 female patients with vitiligo. Female patients showed an early age of onset of disease as compared to male patients (Mean age of onset ± SEM: 19.20 ± 1.091 vs 28.08 ± 1.796; p<0.0001).

7.3.6 Relative gene expression of *NALP1* in patients with vitiligo and controls:

Comparison of the findings showed significant increase in expression of *NALP1* transcripts in 122 vitiligo patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p<0.0001) (Figure 4A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.132 fold change in the expression of *NALP1* transcript in patients as compared to controls (Figure 4B).

7.3.6.1 Correlation of *NALP1* transcripts with the investigated polymorphisms:

Further, the expression levels of *NALP1* were analyzed with respect to A/G (rs2670660) promoter genotypes (Figure 4C). Interestingly, *NALP1* expression was significantly increased in patients with susceptible GG genotypes as compared to controls (p=0.001). Also, patients with genotypes AG showed increased *NALP1* transcripts as compared to controls (p=0.007); however, no significant difference was observed in *NALP1* expression in patients as compared to controls with AA genotypes (p=0.102).

The genotype-phenotype correlation for T/C (rs6502867) showed significant increased expression of *NALP*1 with CC and TC genotypes in patients as compared to those of controls (p=0.020; p=0.006); however, no significant difference was observed in *NALP1* expression in patients as compared to controls with TT genotypes (*p*=0.121) (Figure 4D). The genotype-phenotype analysis for A/T (rs12150220) SNP did not show any significant difference in *NALP*1 expression between patients and controls (p=0.082; p=0.162; p=0.160) (Figure 4E).

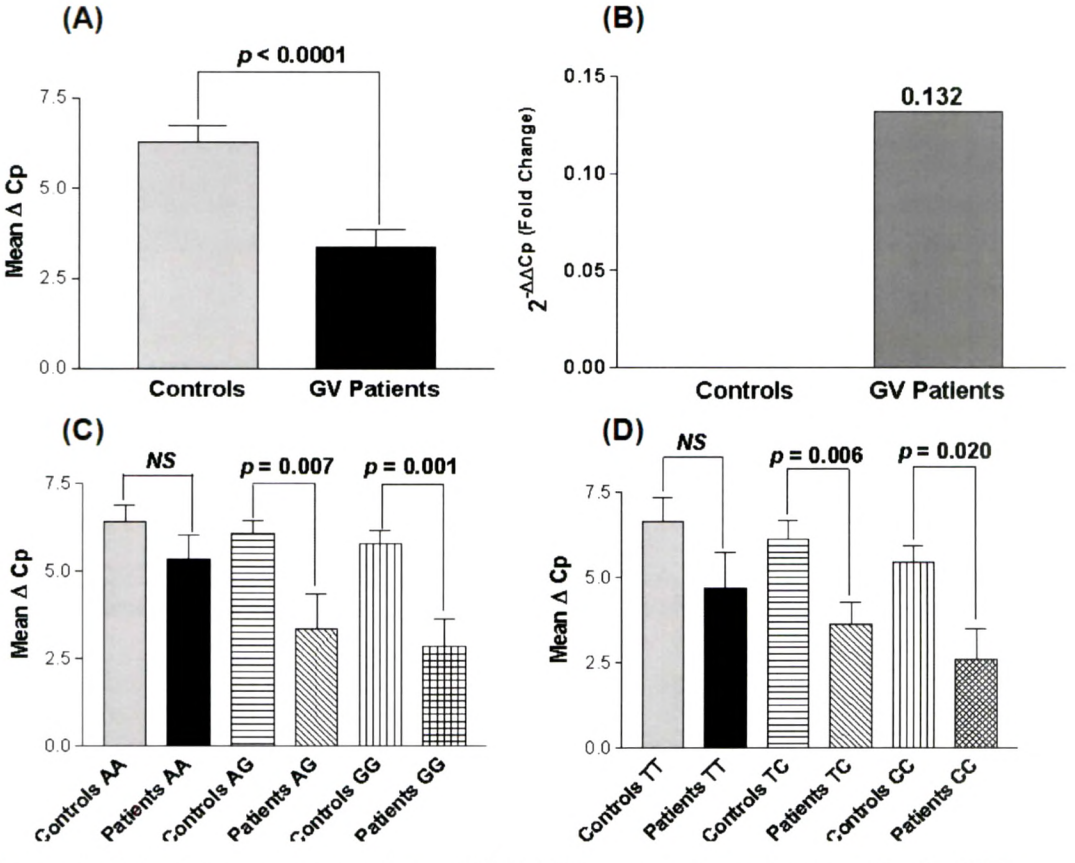


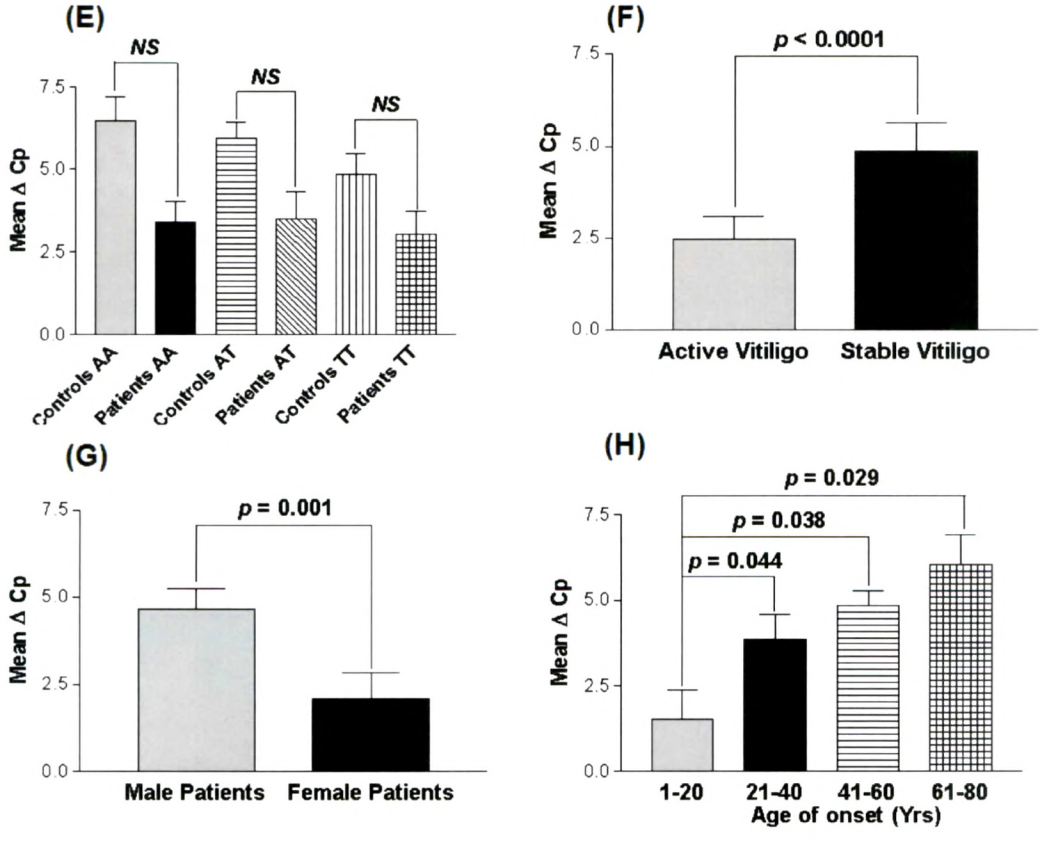
Figure 4. Relative gene expression of *NALP1* in controls and generalized vitiligo patients:

(A) Expression of *NALP1* transcripts in 175 controls, 122 generalized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *NALP1* as compared to controls (Mean ΔCp ± SEM: 3.360 ± 0.5046 vs 6.276 ± 0.4784; p<0.0001).

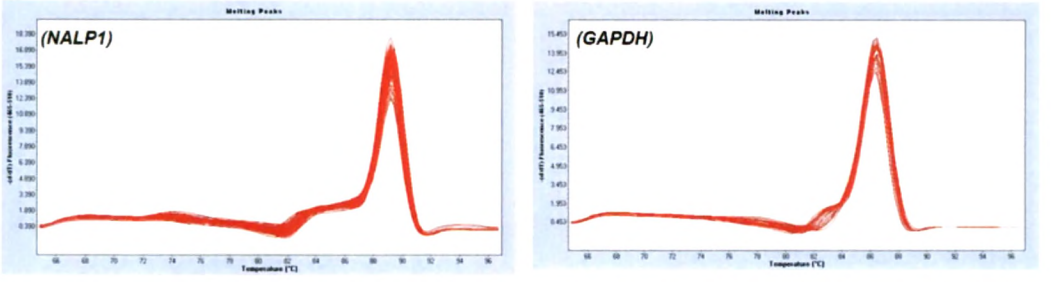
(B) Expression fold change of *NALP1* transcripts in 122 vitiligo patients against 175 controls showed 0.132 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *NALP1* transcripts with respect to *NALP1* A/G (rs2670660) promoter polymorphism in 122 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *NALP1* with GG (Mean ΔCp ± SEM: 2.841 ± 0.7811 vs 5.796 ± 0.3632; p=0.001) and AG (Mean ΔCp ± SEM: 3.348 ± 1.000 vs 6.065 ± 0.3999; p=0.007) genotypes as compared to controls. There was no significant difference in the expression of *NALP1* in patients with AA genotypes (Mean ΔCp ± SEM: 5.357 ± 0.6911 vs 6.712 ± 0.4496; p=0.102) as compared to controls. [*NS* = non-significant]

(D) Expression of *NALP1* transcripts with respect to *NALP1* T/C (rs6502867) intron polymorphism in 122 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *NALP1* with CC (Mean ΔCp ± SEM: 2.598 ± 0.9118 vs 5.434 ± 0.4912; p=0.020) and TC (Mean ΔCp ± SEM: 3.622 ± 0.6535 vs 6.125 ± 0.5546; p=0.006) genotypes as compared to controls. There was no significant difference in the expression of *NALP1* in patients with TT genotypes (Mean ΔCp ± SEM: 4.671 ± 1.079 vs 6.639 ± 0.6960; p=0.121) as compared to controls.



(I)



(E) Expression of *NALP1* transcripts with respect to *NALP1* A/T (rs12150220) intron polymorphism in 122 vitiligo patients and 175 controls, as suggested by Mean ΔCp. There was no significant difference in mRNA levels of *NALP1* in patients with TT (Mean ΔCp ± SEM: 3.040 ± 0.6779 vs 4.854 ± 0.6088; p=0.082), AT (Mean ΔCp ± SEM: 4.153 ± 1.025 vs 5.931 ± 0.4993; p=0.162) and AA (Mean ΔCp ± SEM: 4.753 ± 0.9490 vs 6.456 ± 0.7339; p=0.160) genotypes as compared to controls.

(F) Expression of *NALP1* transcripts with respect to activity of the disease in 91 patients with active vitiligo and 31 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients showed significantly increased mRNA levels of *NALP1* as compared to stable vitiligo patients (Mean ΔCp ± SEM: 2.472 ± 0.6268 vs 4.866 ± 0.7669; p=0.021).

(G) Expression of *NALP1* transcripts with respect to gender differences in 52 male patients and 70 female patients with vitiligo, as suggested by Mean ΔCp. Female patients with vitiligo showed significantly increased mRNA levels of *NALP1* as compared to male vitiligo patients (Mean ΔCp ± SEM: 4.649 ± 0.5885 vs 2.071 ± 0.7604; p=0.001).

(H) Expression of *NALP1* transcripts with respect to different age groups in 122 vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients with the age of onset 1-20 yrs showed significantly increased expression of *NALP1* mRNA as compared to the age groups 21-40 yrs (Mean ΔCp ± SEM: 1.516 ± 0.8508 vs 3.853 ± 0.7290; p=0.044), 41-60 (Mean ΔCp ± SEM: 1.516 ± 0.8508 vs 4.841 ± 0.4153; p=0.038) and 61-80 yrs (Mean ΔCp ± SEM: 1.516 ± 0.8508 vs 6.028 ± 0.8713; p=0.029).

(I) Melt curve analysis of *NALP1* and *GAPDH* showing specific amplification.

7.3.6.2 Effect of *NALP1* expression on disease progression:

In addition, we also checked the effect of *NALP1* expression on progression of the disease i.e. active and stable cases (Figure 4F). Interestingly, active vitiligo patients showed significant increase in expression of *NALP1* transcripts as compared to the patients with stable vitiligo (p=0. 021) suggesting the involvement of *NALP1* in disease progression. Moreover, the susceptibility of the disease was also checked based on the gender differences and we found that female patients with vitiligo showed significantly higher *NALP1* expression as compared to male patients (p=0. 001) (Figure 4G).

When *NALP1* expression was monitored in different age of onset groups of patients, patients with the age group 1-20 yrs showed significantly increased expression of *NALP1* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.044, p=0.038 and p=0.029 respectively) suggesting the importance of *NALP1* in early onset of the disease (Figure 4H).

7.4 DISCUSSION

The locus for familial vitiligo on chromosome 17 harbours the gene coding for *NALP1* (Jin *et al.*, 2007a). The protein contains a NACHT domain, LRR domain (leucine rich repeat) and a pyrin domain. NALP1 protein is a component of a large signal induced cytoplasmic multiprotein complex called inflammasome, which is a direct sensor of bacterial infections (Kummer *et al.*, 2007). NALP1 contains a caspase recruitment domain which is known to be the key mediator of apoptosis. The N-terminal pyrin like motif is involved in protein-protein interaction which interacts strongly with caspase-2 caspase-9 and apoptotic protease activating factor-1 (Apaf-1) (Faustin *et al.*, 2007; Bonfoco *et al.*, 2001).

NALP1 is known to be involved in inflammation and apoptosis (Martinon *et al.*, 2001; Tschopp *et al.*, 2003). NALP1 may also contribute to modulate the response of cells towards proinflammatory stimuli like IL-1β, IFN-γ and TNF-α. Overexpression of this gene induces apoptosis in cells (Kummer *et al.*, 2007). Even transient overexpression of *NALP1* in *in vitro* cultured cells was sufficient to induce apoptosis. The primary function of human NALP1 is caspase activation. Upon activation by

appropriate pathogen associated molecular pattern (PAMP)s, resulting in its oligomerization, human NLRP1 binds adapter protein apoptosis associated speck like protein containing CARD (ASC) via its N-terminal PYRIN-PAAD-DAPIN domain (PYD). ASC, in turn, binds pro-caspase-1, forming the inflammasome (Martinon *et al.*, 2002). The C-terminal CARD of NALP1 can also bind both pro-caspase-1 and pro-caspase-5 (Tschopp *et al.*, 2003). These members of the caspase family activate IL-1β and IL-18, as well as cause apoptosis.

NALP1 may have other functions besides activation of caspase-1 in response to bacterial PAMP, muramyl dipeptide (MDP). For example, NLRP1 was reported to bind the patched receptor complex via adapter protein DRAL, stimulating caspase-9 activation (Mille *et al.*, 2009). In this regard, the CARD of NALP1 binds the CARD of Apaf-1, a caspase-9 activating protein that becomes activated by cytochrome c when released from mitochondria (Bruey *et al.*, 2007; Chu *et al.*, 2001). Thus, it is possible that NALP1 contributes to apoptosis via caspase-9 activation in some contexts. Also, it is possible that Apaf1 and components of patched receptor complex are intrinsic activators of NALP1. Cytochrome c-mediated oligomerization of Apaf-1 conceivably could create a platform for recruitment of NALP1, resulting in caspase-1 activation via a mechanism whereby the N-terminal PYD domain of NALP1 binds ASC, which in turn binds pro-caspase-1, while the C-terminal CARD of NRLP1 binds the CARD of Apaf1. Activated patched receptors also could theoretically create an oligomerized protein complex at the plasma membrane for NALP1 recruitment, thus promoting caspase activation.

NALP1 is widely expressed at low levels in all cells but is expressed at high levels in blood mononuclear cells and immune cells, particularly granulocytes, monocytes, T-lymphocytes and Langerhans cells. Immunohistochemical studies have found high *NALP1* expression in epidermis within Langerhans cells (Kummer *et al.*, 2007) suggesting involvement of NALP1 in skin autoimmunity. NALP1 is part of inflammasomes that regulate the activation of caspases which in turn convert proinflammatory cytokines into their active forms. Taieb (2007) suggested that NALP1 could help trigger or enhance the autoimmune part of vitiligo, which may involve initial contacts between melanocytes and NALP1-positive Langerhans.

Earlier, several SNPs in *NALP1* have been studied with vitiligo susceptibility; however, only a few of them achieved significant association with vitiligo (Jin *et al.*, 2007a,b; Alkhateeb, 2010). The functional significance of these SNPs is not yet known, but they are proposed to have a regulatory effect on *NALP1* expression. The A/G (rs2670660) SNP is present in *NALP1* promoter region which is conserved in human, chimpanzee, macaque, bush baby, cow, mouse and rat, suggesting that this variant is functionally significant. It alters predicted binding motifs for the transcription factors high mobility group protein (HMGA1) [HMG-I(Y)] and MYB. MYB regulates transcription during the differentiation, proliferation, and apoptosis of erythroid, myeloid, and lymphoid cell lineages. The structural polymorphism A/T (rs12150220) is a non-synonymous coding SNP which substitutes Leucine at 155 position toHistidine. The amino acid sequence, including Leu155, is highly conserved throughout primate evolution, suggesting that this region is critical for protein function. The other T/C (rs6502867) SNP is located in an intron towards the 3' end of the *NALP1* gene.

Variations in the *NALP1* gene have been reported to confer risk for vitiligo and extended autoimmune disorders in Caucasian patients from the United Kingdom, the United States and Romania (Jin *et al.*, 2007a, b). This study confirms genetic association of generalized vitiligo with variation in *NALP1*, which contains at least two independent risk signals, one tagged by SNP rs2670660 and another tagged by SNPs rs2670660 and rs8182352. A report from cohort of Arab vitiligo patients suggested significant association of two SNPs in the *NALP1* extended promoter region: rs1008588 and rs2670660 with generalized vitiligo (Alkhateeb and Qarqaz, 2010). Our present study focuses on identification of genetic variants of *NALP1* which were significantly associated with vitilgo susceptibility in earlier studies. Intersetingly, we also found significant association of *NALP1* rs2670660 (A/G) and rs6502867 (T/C) SNPs with vitiligo susceptibility in Gujarat population; however rs12150220 (A/T) polymorphism was not associated with vitiligo susceptibility (Table 3). In particular, the 'G' allele of promoter polymorphism (A/G; rs2670660) which may be involved in regulation of *NALP1* expression was found to be prevalent in vitiligo especially in active cases of vitiligo which signifies that NALP1 also modulates the disease preogression. Moreover, the minor allele 'G' was prevalent in female patients compared to male patients suggesting the inclination of autoimmune

preponderance in females (Table 5). Another SNP rs2670660 (T/C) present in the intronic region of *NALP1* was also found to be in significant association with generalized vitiligo, active cases of vitiligo and with female vitiligo patients (Table 3, 4 & 5).

The present study found significant increase in expression of *NALP1* transcripts in vitiligo patients (Figure 4A). The expression of *NALP1* was also increased in active cases of vitiligo as compared to patients with stable vitiligo (Figure 4F). Also, female patients had increased expression of *NALP1* as compared to male patients with vitiligo suggesting that females are more inclined to autoimmunity (Figure 4G). The *NALP1* expression was also increased in patients with early onset of vitiligo as compared to those of late onset suggesting the important role of NALP1 in early phase of the disease (Figure H). In support of this, age of onset analysis with different genotypes of *NALP1* polymorphisms such as A/G (rs2670660) and T/C (rs6502867) revealed that patients harboring susceptible genotypes had an early onset of vitiligo as compared to those with wild type genotypes (Figure 3A & B).

Furthermore, the genotype-phenotype correlation for *NALP1* A/G (rs2670660) SNP revealed increased expression of *NALP1* in patients with GG and AG genotypes as compared to those of controls indicating that 'G' allele may be involved in the increased expression of *NALP1* (Figure 4C). Moreover, genotype-phenotype analysis of *NALP1* T/C (rs6502867) SNP showed increased expression of *NALP1* in patients with CC and TC genotypes as compared to those of controls, suggesting the important role of 'C' allele in increased expression of *NALP1* (Figure 4D). However, the non-synonymous A/T (rs12150220) SNP did not reveal any significant correlation of its genotype with the *NALP1* mRNA expression (Figure 4E). Furthermore, the frequency of susceptible haplotype 'GCT' was significantly increased in vitiligo patients as compared to controls suggesting the important role of *NALP1* polymorphisms in susceptibility of generalized vitiligo.

Nevertheless, the exact functional consequences of these polymorphic variants remain to be determined. The complexity of NALP1 regulation and its various putative effector mechanisms (inflammation, apoptosis, etc.) raise several interesting possibilities for how the sequence variations in NALP1 might impact immune system

function. The current study attempted to elucidate the role of these variants; however, further investigations are needed to elaborate the mechanistic insights that might reveal functional consequences of *NALP1* variants in vitiligo and other autoimmune disorders. In conclusion, our findings suggest that the increased *NALP1* expression in vitiligo patients could result, at least in part, from variations at the genetic level. The study also emphasizes the influence of *NALP1* on the disease progression, onset of the disease and gender biasness for developing vitiligo. More detailed studies regarding the role of *NALP1* in precipitation of vitiligo may prove to be useful for development of effective preventive/ameliorative therapies.

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CHAPTER VIII

DETERMINATION OF $CD4^+/CD8^+$ RATIO AND EVALUATION OF $CD4^+CD25^{hi}FOXP3^+$ T-REGULATORY CELLS (Tregs) AND THEIR RELATION TO EFFECTOR T-CELLS (Teffs) IN GENERALIZED VITILIGO PATIENTS AND CONTROLS

8.1 INTRODUCTION

Vitiligo is an acquired depigmentation disorder characterized by loss of melanocytes from the epidermis. This condition affects approximately 0.5–1% of the world population (Taieb *et al.,* 2007). The exact etiology of vitiligo remains obscure, but autoimmunity has been strongly implicated in the development of disease, especially in generalized vitiligo (GV), because approximately 30% of vitiligo patients are affected with at least one additional autoimmune disorder (Alkhateeb *et al.,* 2003).
In addition, circulating autoantibodies (Cui and Bystryn, 1995; Kemp *et al.,* 1997), autoreactive $CD8^+$ cytotoxic T-cells and macrophages (Ogg *et al.,* 1998; Lang *et al.,* 2001; Le Gal *et al.,* 2001; Mandelcorn-Monson *et al.,* 2003; Wijngaard *et al.,* 2000) that recognize pigment cell antigens have been detected in the sera of a significant proportion of vitiligo patients compared with healthy individuals. In particular, active cases of vitiligo were demonstrated to have higher levels of autoantibodies and cytotoxic T-cells (Le Poole and Luiten, 2008). The high frequencies of melanocyte-reactive cytotoxic T cells in the peripheral blood of vitiligo patients, peri-lesional T-cell infiltration and melanocyte loss *in situ* suggest the important role of cellular autoimmunity in the pathogenesis of this disease. In most vitiligo patients the balance of cytotoxic/suppressor and helper/inducer T-cells in peripheral blood is disturbed which might lead to predominance of distinct T-cell subtypes. Moreover, in progressive disease, the $CD4^+/CD8^+$ ratio is decreased among skin-infiltrating T cells and $CD8^+$ T cells isolated from vitiligo skin are cytotoxic to melanocytes (Wankowicz-Kalinska *et al.,* 2003). Normal values of $CD4^+$ and $CD8^+$ cells are approximately 30-60% and 10-30% respectively in healthy individuals depending on age ($CD4^+/CD8^+$ ratio: 0.9 to 3.7 in adults) (Reniz *et al.,* 1987).

Cytotoxic T-cells require $CD4^+$T-cell help to be activated against melanocytes (Steitz *et al.,* 2005). Involved helper and cytotoxic T-cells from progressing vitiligo margins generate predominantly type 1 cytokines, namely, IFN-γ and TNF-α suggesting vitiligo is a Th1-mediated disease (Le Poole *et al.,* 2004). The underlying causes for autoimmune diseases are not yet fully elucidated but autoimmunity may result from unchecked control by regulatory T-cells (Tregs) (Levings *et al.,* 2001).

A number of T-cell subsets with immunoregulatory activities have been described, and their roles in certain animal autoimmune disease models have been shown and are likely to be of relevance in human immune-mediated diseases (Maloy *et al.,* 2001). Regulatory T cells are critical for the maintenance of immune cell homeostasis. Specifically, Treg cells maintain order in the immune system by enforcing a dominant negative regulation on other immune cells. Accumulating data indicate that a deficiency or dysfunction of Tregs is associated with impaired immune homeostasis and the development of autoimmune diseases. $CD4^+CD25^+FoxP3^+$ regulatory T cells are important in maintaining self-tolerance and regulating immune responses in both physiological and pathological conditions (Sakaguchi, 2004). These naturally occurring T cells can control actively and dominantly the activation and function of autoreactive T cells that have escaped from the thymus and can prevent development of the autoimmune disease.

$CD4^+CD25^+FoxP3^+$ Tcell subset constitutively expresses high levels of CD25, the IL-2R α-chain, in addition to CD4 (Takahashi *et al.,* 1998). This population constitutes about 4-10% of peripheral $CD4^+$ cells and shows remarkable suppressive capacity both *in vitro* and *in vivo* (Suri-Payer *et al.,* 1998; Itoh *et al.,* 1999; Sasaki *et al.,* 2004). Since CD25 is also expressed in activated T-helper cells, FoxP3 was found to be a more specific marker for Tregs. FoxP3 is the acronym given to the forkhead winged-helix transcription factor "forkhead box P3" which serves as the dedicated mediator of the genetic program governing Tregs development and function, therefore it is regarded as a reliable and specific marker for Tregs (Hori *et al.,* 2003; Yagi *et al.,* 2004). Other markers expressed by Treg include GITR and CTLA-4 yet only FoxP3 expression is relatively unique to regulatory T cells (De Boer *et al.,* 2007). The transcription factor 'FoxP3' affects the expression of many genes (Zheng and Rudensky, 2007), and mutations in FoxP3 can cause severe autoimmune disorders as in immune dysregulation, polyendocrinopathy, Enteropathy X-linked (IPEX) syndrome (human) and scurfy mice, supporting the importance of Treg to keep autoreactive T cells in check (Lahl *et al.,* 2007).

$CD4^+CD25^{hi}FoxP3^+$ Tregs are further subdivided into natural, thymically derived Tregs and adaptive Tregs. Inhibition of effector cells proceeds via cell-cell contact by the former subset and via the secretion of inhibitory cytokines, mainly TGF-β by the

latter. After T-cell receptor (TCR)-mediated stimulation, $CD4^+CD25^{hi}FoxP3^+$ Tregs suppress the activation and proliferation of other $CD4^+$ and $CD8^+$ T-cells in an antigen non-specific manner (Thornton and Shevach, 2000; Thornton *et al.*, 2006).

Recently, the level of $CD4^+CD25^{hi}FoxP3^+$ Tregs and their relation to effector T-cells (Teffs) have been evaluated in generalized vitiligo demonstrating the elevation of Tregs and Teffs (Abdallah and Saad, 2009). In contrast, another study by Lili *et al.* (2012) indicated that reduced numbers and impaired function of natural Tregs fail to control the widespread activation of $CD8^+$ CTLs, which leads to the destruction of melanocytes in GV. Klarquist *et al.* (2010) showed a defect in Treg cell homing to the skin, based on the finding of drastically reduced Treg numbers in vitiligo skin without any systemic drop in their abundance or activity. However, Ben Ahmed *et al.*, identified increased number of Tregs in perilesional skin despite a functional defect of circulating Tregs in progressive vitiligo (Ben Ahmed *et al.*, 2011). Thus whether the prevalence and/or function of Tregs are truly impaired in GV patients is still controversial.

The aim of the present study was to evaluate $CD4^+/CD8^+$ ratio and $CD4^+CD25^+FoxP3^+$ Tregs and their relation to (Teffs) in patients with generalized vitiligo and compare them to unaffected controls.

8.2 MATERIALS AND METHODS

8.2.1 Patients and controls

The study group included 82 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 39 males and 43 females who referred to S.S.G. Hospital, Vadodara, India (Table 1). The patients were divided into two groups based on whether the existing lesions were spreading and/ or new lesions had appeared within the previous 6 months: an affirmative answer to one or both of those questions led to inclusion of the patient in the progressive GV group, whereas patients with no increase in lesion size or number were included the stable GV group. Fifty-six patients were classified with progressive GV, whereas twenty-nine patients were included in the stable GV group. The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated

autoimmune diseases. A total of 50 sex-matched unaffected individuals (24 males and 26 females) were included as controls in the study (Table 1). Demographic data for patients and controls are shown in Table 1. None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained in writing from all subjects before performing the studies.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 82)	(n = 50)
Average age (mean age ± SD)	34.28 ± 18.95 yrs	30.41 ± 11.93 yrs
Sex: Male	39 (47.56%)	24 (48.00%)
Female	43 (52.44%)	26 (52.00%)
Age of onset (mean age ± SD)	26.69 ± 19.01 yrs	NA
Duration of disease (mean ± SD)	7.35 ± 8.51 yrs	NA
Active vitiligo	56 (68.29%)	NA
Stable vitiligo	29 (31.71%)	NA
Family history	11 (13.41%)	NA

8.2.1.1 Determination of $CD4^+/CD8^+$ ratio in vitiligo patients and controls of Gujarat

Flow cytometry

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD Biosciences, San Jose, CA). The blood samples were processed immediately within 2 h of collection, for determining the counts of $CD4^+$, $CD8^+$ cells and their ratios by two colour immunophenotyping on FACS AriaIII system (BD Biosciences, San Jose, CA), using fluorochrome labeled monoclonal antibodies to $CD4^+$ and $CD8^+$ T-cells, strictly following manufacturer's instructions (BD Simultest CD4/CD8 Reagent). The same batches of antibodies were used for testing the samples of subjects of all the groups. 100 µl of whole anticoagulated blood was lysed using 1 mL (1X) FACS Lysing Solution (BD Biosciences, San Jose, CA) followed by washing with phosphate buffer saline (PBS). After that, the cells were stained with combinations of the following antibodies (20 µl each): anti-CD4-FITC, anti-CD8-PE. The tubes were then incubated in the dark for 30 minutes followed by washing with PBS containing 0.1% sodium azide, fixed in PBS containing 1% paraformaldehyde, and acquired the same day on a FACS AriaIII system (BD Biosciences, San Jose, CA) followed by analysis using FlowJo software version 7.6.5 (Tree Star, San Carlos, CA). A doublet discrimination strategy was performed for the analysis of $CD4^+$ and $CD8^+$ T-cells (Figure 1).

8.2.1.2 Evaluation of $CD4^+CD25^{hi}FoxP3^+$ T-regulatory cells (Tregs) and their relation to effector T-cells (Teffs) in vitiligo patients and controls of Gujarat

Flow cytometry

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD Biosciences, San Jose, CA). All blood samples were processed immediately within 2 h of collection. Peripheral blood mononuclear cells (PBMCs) were purified on Ficoll gradients (GE Healthcare, Bjo¨rkgatan, Sweden). For the detection of natural Treg cells, PBMCs were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD25, and phycoerythrin (PE)-conjugated anti-FoxP3 (BD Biosciences, San Jose,

CA), according to the manufacturer's protocol. Initially, surface staining of CD4 and CD25 was done with 20 µl of respective antibodies followed by incubation of 30 min. at room temperature. For intracellular staining of FoxP3-PE (BD Biosciences, San Jose, CA): cells were fixed and permeabilized with the use of Human FoxP3 Buffer Set (BD Biosciences, San Jose, CA) according to the manufacturer's instructions with certain modifications, in brief: After washing, the cell pellet was resuspended in 0.5 ml of freshly prepared 1X fixation/ permeabilization working solution and incubated for 30 minutes at room temperature in the dark.

After staining, cells were washed twice in FACS staining buffer [phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide], fixed in PBS containing 1% paraformaldehyde, and acquired the same day on a FACS AriaIII system (BD Biosciences, San Jose, CA) followed by analysis using FlowJo software version 7.6.5 (Tree Star, San Carlos, CA). A doublet discrimination strategy was performed for the analysis of Treg cells (Figure 2).

$CD25^{hi}FoxP3^{+}$ natural Treg cells were identified within gated CD4+ T cells. To discriminate between $CD25^{hi}$ Treg and $CD25^{low}$ activated effector T-cells, we used CD25 expression on $CD8^{+}$ cells as an internal control. $CD8^{+}$ cells only express intermediate levels of CD25 ($CD25^{low}$), whereas $CD4^{+}$ T-cells express CD25 with high ($CD25^{high}$) or intermediate ($CD25^{low}$) intensities. Only $CD4^{+}$ cells expressing CD25 with higher intensities than the $CD8^{+}$ cells were included in the gate for $CD25^{high}$ cells. The gate for $CD25^{low}$ cells was set to include cells expressing CD25 at levels above those of the isotype control and unstained cells but at lower expression levels than the $CD25^{high}$ cells (Figure 5).

8.2.2 Statistical analysis

Data analysis was performed using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). Quantitative data were expressed as the mean ± SEM. Statistical significance was determined by unpaired Student's t-test. *p*-values less than 0.05 were considered as statistically significant.

8.3 RESULTS

8.3.1 Determination of $CD4^+/CD8^+$ ratio in vitiligo patients and controls from Gujarat

$CD4^+$ and $CD8^+$ T-cells were identified in vitiligo patients and controls by FACS analysis as shown in Figure 1. The mean $CD4^+$ and $CD8^+$ T-cell counts in vitiligo patients and controls are given in Table 2. The $CD8^+$ cell counts were significantly higher in vitiligo patients as compared to controls (p=0.003); however, $CD4^+$ cell counts did not show significant difference between patients and controls (p=0.328) (Figure 2A). As the mean cell count of $CD8^+$ T-cell significantly increased, $CD4^+/CD8^+$ ratio decreased significantly in patients as compared to controls (Figure 2B). Analysis based on the stage of progression of vitiligo suggested increased $CD8^+$ T-cell count in patients with active vitiligo as compared to stable vitiligo (p=0.001) (Figure 3B) (Table 3). However, the mean $CD4^+$ T-cell count did not differ between active and stable vitiligo cases (p=0.579) (Figure 3A). The $CD4^+/CD8^+$ ratio in active vitiligo cases significantly lowered as compared to stable vitiligo (p=0.002) suggesting the important role of $CD8^+$ T-cell in progression of the disease (Figure 3C). Moreover, the $CD4^+$ and $CD8^+$ T-cell counts did not differ between male and female patients and hence the $CD4^+/CD8^+$ ratio did not differ (Figure 4A, B & C) suggesting there is no effect of gender biasness in $CD4^+$ and $CD8^+$ T-cell counts in patients.

When $CD4^+/CD8^+$ ratio was monitored in different age of onset groups of patients, patients with the age group 1-20 yrs did not show significant difference as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.267, p=0.318 and p=0.497 respectively) (Table 4).

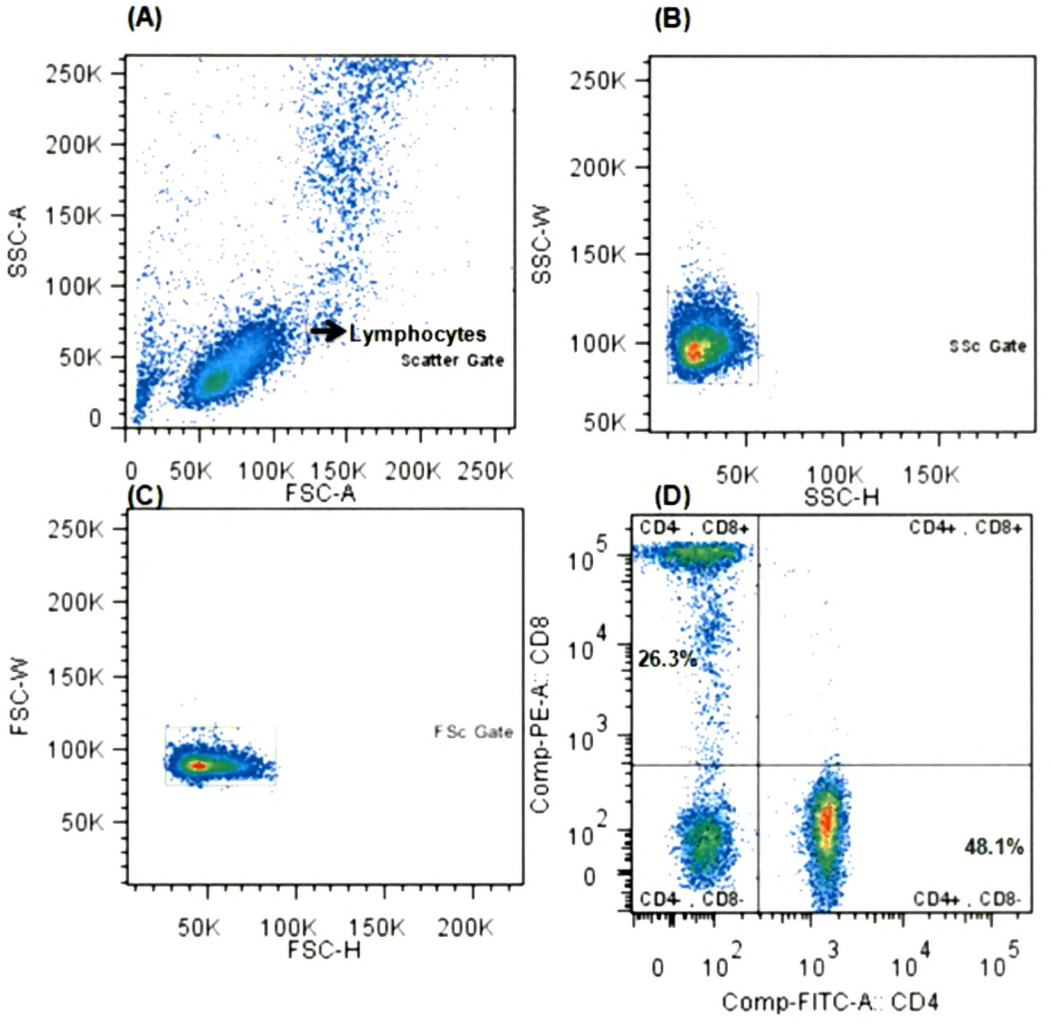


Figure 1. Human $CD4^+$ and $CD8^+$ T-cell subsets identified by FACS analysis (representative FACS plots for $CD4^+$ and $CD8^+$ T-cell):

(A) Lymphocte gating is shown in a scatter gate.

(B) & **(C)** show SSC and FSC plots for doublet discrimination of the lymphocyte population.

(D) $CD4^+CD8^-$, $CD4^-CD8^+$ cells were identified as shown.

Table 2. Counts of $CD4^+$ and $CD8^+$ T cells and $CD4^+$ /$CD8^+$ ratio in vitiligo patients and controls.

T Cells	Vitiligo Patients (n = 82)		Controls (n = 50)		*p* Value
	Mean ± SEM (no. of cells)	Range	Mean ± SEM (no. of cells)	Range	
$CD4^+$	2984 ± 192.5	706-7482	2603 ± 377.4	602-6911	0.328
$CD8^+$	4080 ± 275.9	458-10967	2588 ± 370.2	678-5478	0.003
$CD4^+/CD8^+$	0.8326 ± 0.0502	0.3162-1.900	1.076 ± 0.0768	0.6183-1.904	0.001

n= number of patients and controls, SEM: standard error mean.

Table 3. Counts of $CD4^+$ and $CD8^+$ T cells and $CD4^+$ /$CD8^+$ ratio in patients with active and stable vitiligo.

T Cells	Active Vitiligo (n = 56)		Stable Vitiligo (n = 29)		*p* Value
	Mean ± SEM (no. of cells)	Range	Mean ± SEM (no. of cells)	Range	
$CD4^+$	2874 ± 224.7	756-4711	3090 ± 312.7	705-7482	0.579
$CD8^+$	4793 ± 449.6	458-10967	3395 ± 269.6	570-5263	0.001
$CD4^+/CD8^+$	0.6790 ± 0.0669	0.3162-1.900	0.98 ± 0.0625	0.5711-1.666	0.002

n= number of patients and controls, SEM: standard error mean.

Table 4. Counts of $CD4^+$ and $CD8^+$ T cells and $CD4^+$ /$CD8^+$ ratio with respect to age of onset of vitiligo.

Age of Onset (yrs)	n	Subsets	Mean ± SEM	Range	95% CI	p Value
1-20	40	$CD4^+$	3033 ± 248.8	756 - 5733	2520 - 3545	-
		$CD8^+$	4042 ± 298.4	458 - 6149	3423 - 4661	-
		$CD4^+$ /$CD8^+$	0.8501 ± 0.07488	0.4789 - 1.900	0.6948 - 1.005	-
21-40	20	$CD4^+$	2958 ± 365.8	706 - 4819	2167 - 3748	0.863[a]
		$CD8^+$	4677 ± 784.2	1056 - 10967	2969 - 6386	0.375[b]
		$CD4^+$ /$CD8^+$	0.7229 ± 0.06943	0.3805 - 1.238	0.5717 – 0.8742	0.267[c]
41-60	16	$CD4^+$	2952 ± 653	745 - 7482	1446 - 4458	0.888[a]
		$CD8^+$	3314 ± 610.5	570 - 5763	1906 - 4722	0.240[b]
		$CD4^+$ /$CD8^+$	0.9992 ± 0.1370	0.4407 – 1.666	0.6833 – 1.315	0.318[c]
61-80	6	$CD4^+$	2831 ± 744.4	1414 - 4323	461.3 - 5200	0.773[a]
		$CD8^+$	4077 ± 332.4	3446 - 4805	3019 - 5135	0.963[b]
		$CD4^+$ /$CD8^+$	0.7132 ± 0.2077	0.3162 – 1.254	0.05212 – 1.374	0.497[c]

[a]Age of onset group 1-20 vs group 21-40,

[b]Age of onset group 1-20 vs group 41-60,

[c]Age of onset group 1-20 vs group 61-80,

n= number of patients, SEM: standard error mean, CI=Confidence Interval.

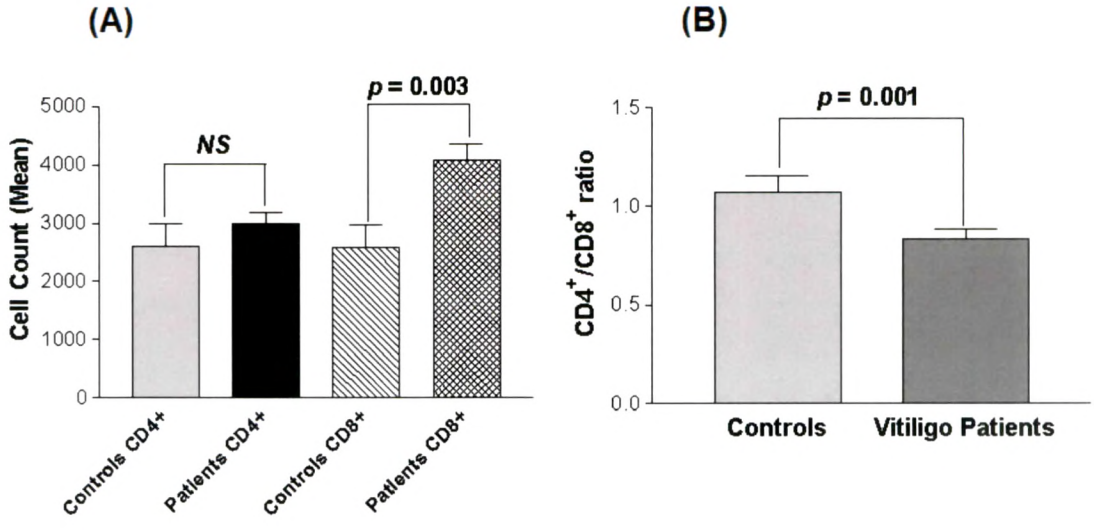


Figure 2. $CD4^+$ and $CD8^+$ T-cell counts and $CD4^+/CD8^+$ ratio in vitiligo patients and controls:

(A) $CD4^+$ and $CD8^+$ T-cell counts in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM for $CD4^+$ controls vs patients (2603 ± 377.4 vs 2984 ± 192.5; p=0.328); $CD8^+$ controls vs patients (2588 ± 370.2 vs 4080 ± 275.9; p=0.003); (NS=non-significant).

(B) $CD4^+/CD8^+$ T-cell ratio in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (1.076 ± 0.0768 vs 0.8326 ± 0.0502; p=0.001).

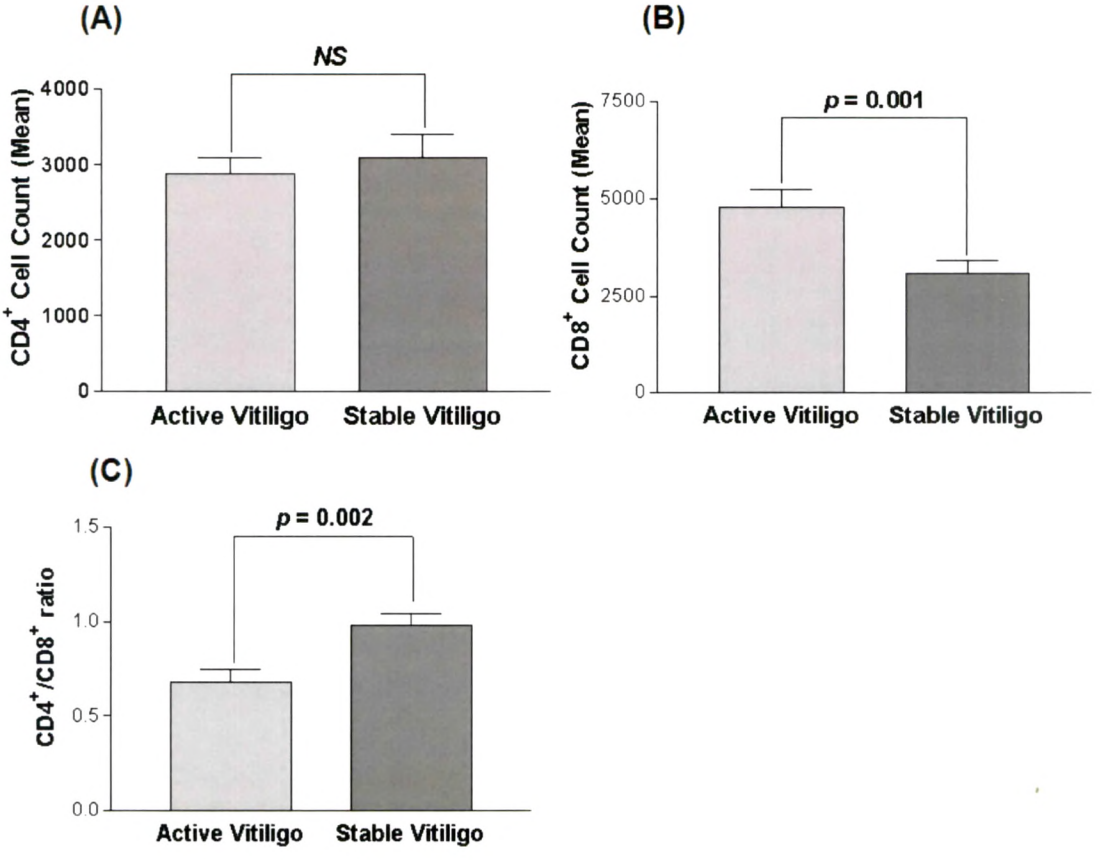


Figure 3. $CD4^+$ and $CD8^+$ T-cell counts and $CD4^+/CD8^+$ ratio in patients with active and stable vitiligo:

(A) $CD4^+$ T-cell counts in active (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: Active vs Stable (2874 ± 224.7 vs 3090 ± 312.7; p=0.579); (NS=non significant).

(B) $CD8^+$ T-cell counts in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (4793 ± 449.6 vs 3395 ± 269.6; p=0.001).

(C) $CD4^+/CD8^+$ T-cell ratio in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (0.6790 ± 0.0669 vs 0.98 ± 0.0625; p=0.002).

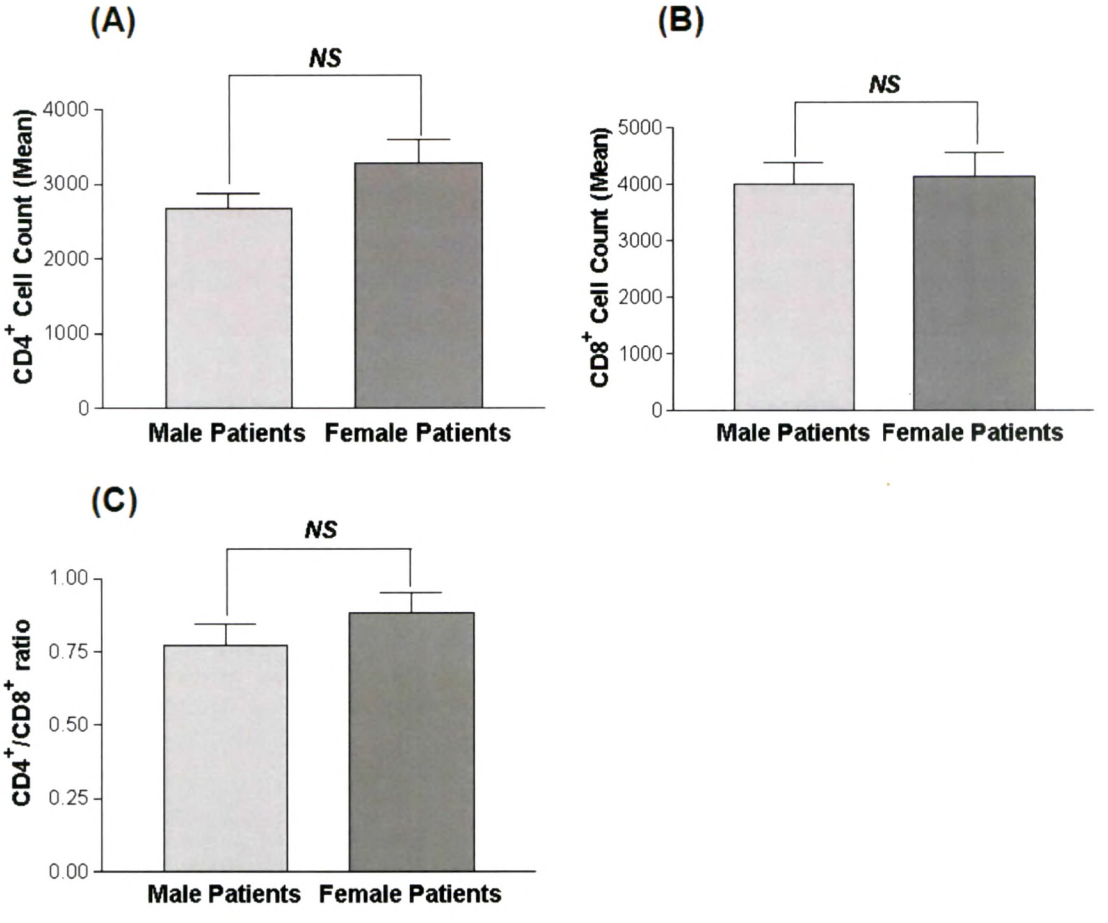


Figure 4. $CD4^+$ and $CD8^+$ T-cell counts and $CD4^+/CD8^+$ ratio in male and female patients with vitiligo:

(A) $CD4^+$ T-cell counts in male (n = 39) and female vitiligo patients (n = 43). Values are given as mean ± SEM: male vs female (2674 ± 210.9 vs 3282 ± 311.9; p=0.116); (NS=non significant).

(B) $CD8^+$ T-cell counts in male (n = 39) and female vitiligo patients (n = 43). Values are given as mean ± SEM: male vs female (4008 ± 372.2 vs 4143 ± 409.1; p=0.811); (NS=non significant).

(C) $CD4^+/CD8^+$ T-cell ratio in male (n = 39) and female vitiligo patients (n = 43). Values are given as mean ± SEM: active vs stable (0.774 ± 0.07217 vs 0.8844 ± 0.06955; p=0.277); (NS=non significant).

8.3.2 Evaluation of $CD4^+CD25^{hi}FoxP3^+$ T-regulatory cells (Tregs) and their relation to effector T-cells (Teffs) in vitiligo patients and controls from Gujarat

8.3.2.1 Defining the human Treg cell population

Using the strict gating parameters reported previously (Baecher-Allan *et al.,* 2001), Tregs were identified as $CD4^+$ cells with higher CD25 expression than that of the $CD4^-$ population (Figure 5E). The frequency of Treg was calculated as the percentage of $CD4^+CD25^{hi}$ cells in the $CD4^+$ population and was in the range of 4.192-12.92% of $CD4^+$ cells in PBMC of healthy age-matched controls. The $CD25^{hi}$ population was further analyzed by the expression of FoxP3 (Figure 5F).

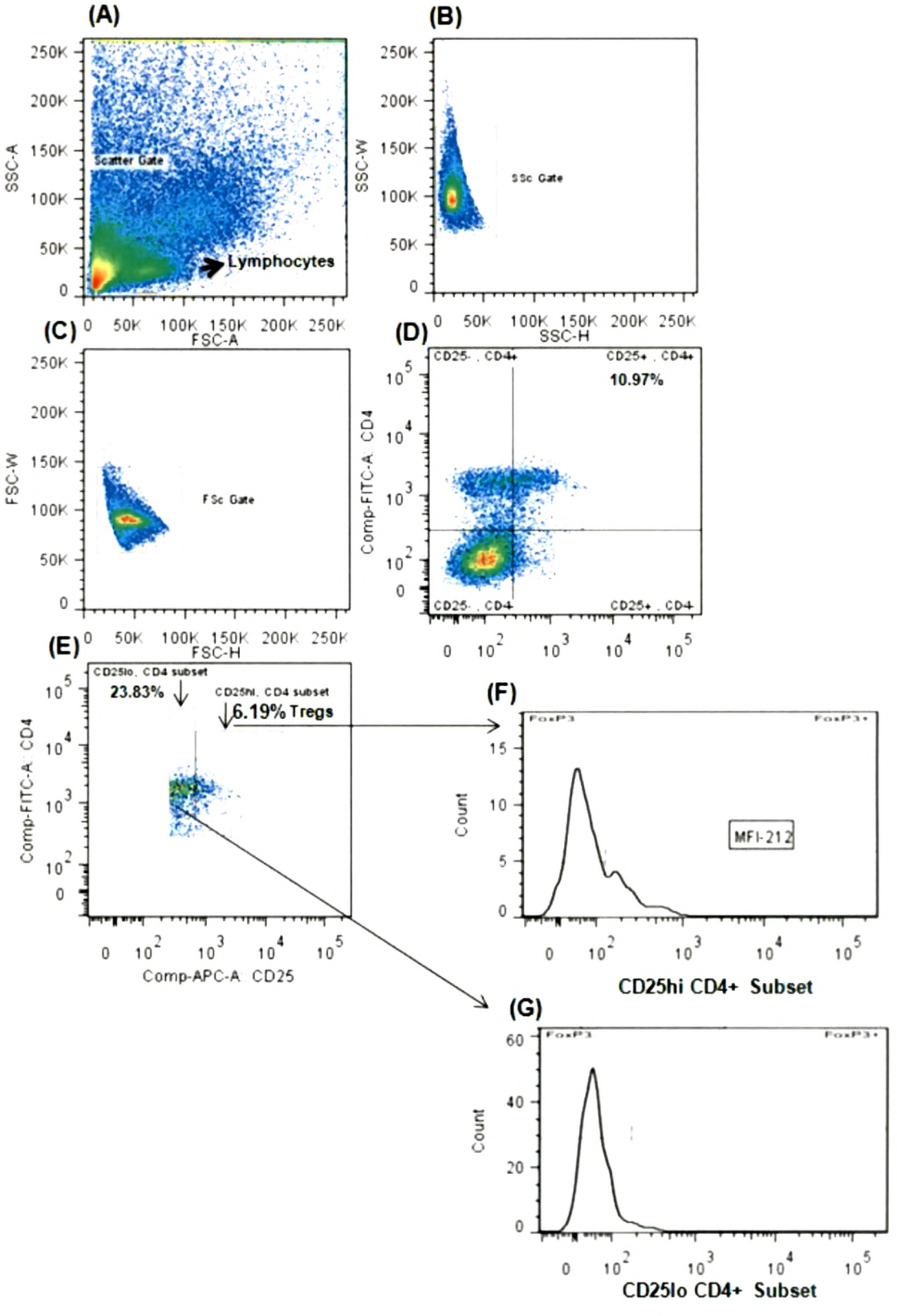

(A)
SSC-A
FSC-A
Scatter Gate
Lymphocytes
(B)
SSC-W
SSC-H
SSc Gate
(C)
FSC-W
FSC-H
FSc Gate
(D)
Comp-FITC-A :: CD4
CD25- , CD4+
CD25+ , CD4+
10.97%
CD25- , CD4-
CD25+ , CD4-
(E)
CD25lo, CD4 subset
23.83%
CD25hi, CD4 subset
6.19% Tregs
Comp-APC-A :: CD25
(F)
FoxP3
FoxP3+
Count
MFI-212
CD25hi CD4+ Subset
(G)
FoxP3
FoxP3+
Count
CD25lo CD4+ Subset

Figure 5. Characterization of human Treg ($CD4^+CD25^{hi}FoxP3^+$) population by FACS analysis (representative FACS plots for Treg ($CD4^+CD25^{hi}FoxP3^+$) cells):
(A) Lymphocte gating is shown in a scatter gate.
(B) & **(C)** show SSC and FSC plots for doublet discrimination of the lymphocyte population.
(D) $CD4^+CD25^-$, $CD4^-CD25^+$ and $CD4^+CD25^+$ cells were identified as shown.
(E) $CD4^+$CD25hi cells are those where CD25 expression was higher than that on the $CD4^-$ population. $CD4^+$CD25lo $FoxP3^-$ (Teffs) population was also identified.
(F) FoxP3 expression within $CD4^+CD25^{hi}$ and $CD4^+CD25^{lo}$ populations was assessed by intracellular staining.

8.3.2.2 Decrease of $CD4^+CD25^{hi}FoxP3^+$ Treg cells in vitiligo patients

There was significant decrease in $CD4^+CD25^{hi}FoxP3^+$ Treg% in generalized vitiligo patients (6.538 ± 0.2863) (range 1.075-9.711) compared to healthy controls (8.122 ± 0.5893) (range 4.192-12.92) (p=0.009) (Figure 6A). FoxP3 expression in Tregs was significantly reduced in $CD4^+CD25^{hi}$ T-cells of patients (592.3 ± 68.03) compared to controls (867.5 ± 52.10) (p=0.024) (Figure 6B).

Analysis based on the stage of progression of vitiligo suggested that percentage of $CD4^+CD25^{hi}FoxP3^+$ Treg cells was significantly decreased in the 56 patients with active cases (6.043 ± 0.4185) compared to 29 patients with stable vitiligo (7.731 ± 0.4334) (p=0.007) (Figure 6C). However, FoxP3 expression in Tregs did not differ between cases of active and stable vitiligo (p=0.779) (Figure 6D). Moreover, gender based analysis of $CD4^+CD25^{hi}FoxP3^+$ Treg cells did not show significant difference in Treg% between male and female patients (p=0.901) (Figure 6E). Interestingly, female patients showed significant decrease in expression of FoxP3 in Tregs (532.7 ± 79.10) as compared to male patients (908.1 ± 137.9) (p=0.021) (Figure 6F).

When $CD4^+CD25^{hi}FoxP3^+$ Treg cells were monitored in different age of onset groups of patients, patients with the age group 1-20 yrs did not show significant difference in Treg% as compared to the age group 21-40 yrs (p=0.793). However, age of onset group 1-20 showed significant decreased Treg% (6.303 ± 0.4351) as compared to age groups 41-60 yrs (8.379 ± 0.5612) and 61-80 yrs (9.610 ± 1.199) (p=0.027 and p=0.010 respectively) (Figure 6G). There was no significant difference in expression

of FoxP3 in Tregs when age of onset group 1-20 yrs (p= 0.060) was compared with age groups 41-60 and 61-80 yrs (p=0.191 and p=0.214 respectively) (Figure 6H).

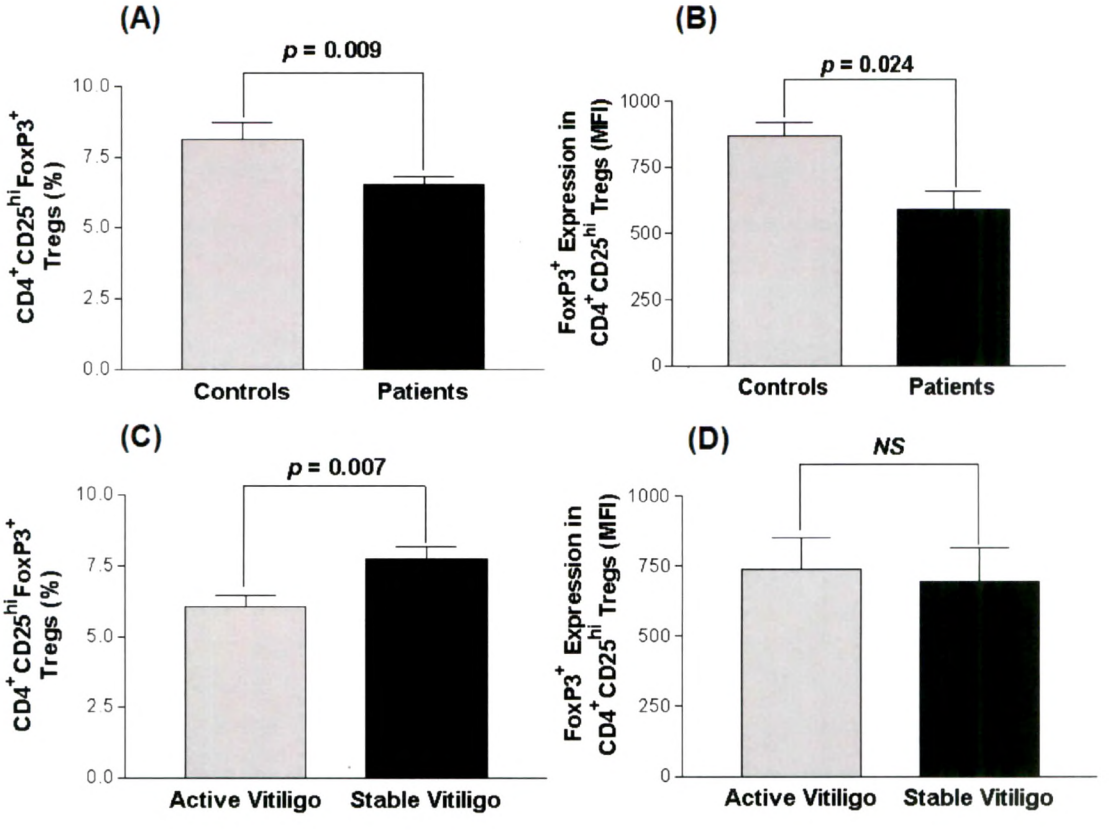


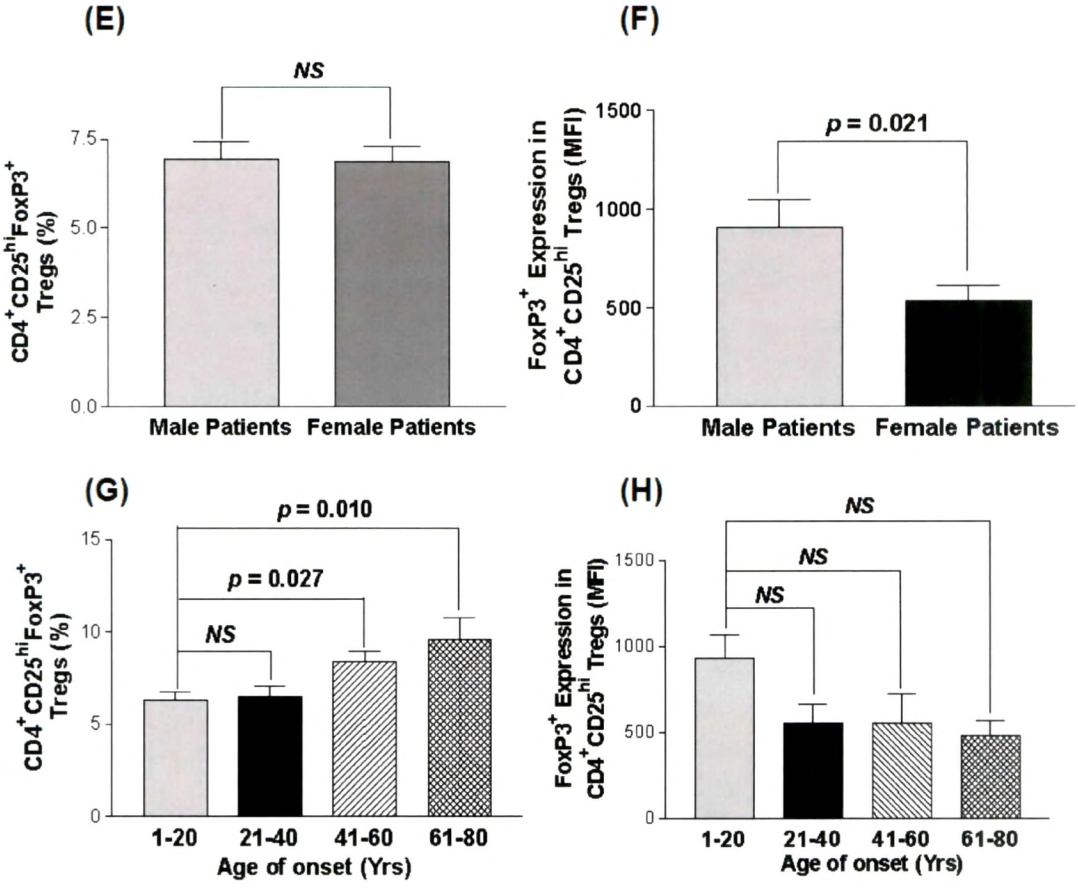
Figure 6. $CD4^+CD25^{hi}Foxp3^+$ Treg cells in vitiligo patients and controls:

(A) $CD4^+CD25^{hi}Foxp3^+$ Treg cells in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (8.122 ± 0.5893 vs 6.538 ± 0.2863; p=0.009).

(B) Foxp3 expression in $CD4^+CD25^{hi}$ Tregs in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (867.5 ± 52.10 vs 592.3 ± 68.03; p=0.024).

(C) $CD4^+CD25^{hi}FoxP3^+$ Treg cells in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (6.043 ± 0.4185 vs 7.731 ± 0.4334; p=0.007).

(D) FoxP3 expression in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (740.7 ± 112.9 vs 693.9 ± 121.1; p=0.779); (MFI=Median Fluorescence Intensity).



(E) $CD4^+CD25^{hi}FoxP3^+$ Treg cells in male (n = 39) and female patients (n = 43) with vitiligo. Values are given as mean ± SEM: male vs female (6.943 ± 0.4768 vs 6.864 ± 0.4396; p=0.903); (NS=non-significant).

(F) FoxP3 expression in $CD4^+CD25^{hi}$ Tregs in male (n = 39) and female patients (n = 43) with vitiligo. Values are given as mean ± SEM: male vs female (908.1 ± 137.9 vs 532.7 ± 79.10; p=0.021); (MFI=Median Fluorescence Intensity).

(G) $CD4^+CD25^{hi}Foxp3^+$ Treg cells in different age of onset groups of patients (1-20 yrs, n = 40; 21-40 yrs, n=20; 41-60 yrs, n=16, 61-80 yrs, n=6). Values are given as mean ± SEM: 1-20 yrs vs 21-40, 41-60, 61-80 yrs (6.303 ± 0.4351 vs 6.495 ± 0.5905, 8.379 ± 0.5612, 9.610 ± 1.199; p=0.793, p=0.027 and p=0.010 respectively).

(H) FoxP3 expression in $CD4^+CD25^{hi}$ Tregs in different age of onset groups of patients (1-20 yrs, n = 40; 21-40 yrs, n=20; 41-60 yrs, n=16, 61-80 yrs, n=6). Values are given as mean ± SEM: 1-20 yrs vs 21-40, 41-60, 61-80 yrs (927.8 ± 136.5 vs 552.6 ± 107.9, 554.9 ± 166.2, 476.8 ± 91.09; p=0.060, p=0.191 and p=0.214 respectively); (MFI=Median Fluorescence Intensity).

8.3.2.3 Increase of T-effector ($CD4^+CD25^{low}FoxP3^-$) cells in vitiligo patients

There was significant increase in $CD4^+CD25^{low}FoxP3^-$ responsive or effector T-cells (Teffs) in vitiligo patients (14.98 ± 0.7445) compared to controls (10.16 ± 1.031) (p=0.001) (Figure 7A). Analysis based on the stage of progression of vitiligo suggested that percentage of $CD4^+CD25^{low}FoxP3^-$ Teff cells was significantly increased in the 56 patients with active cases (13.96 ± 1.010) compared to 29 patients with stable vitiligo (11.09 ± 0.8013) (p=0.029) (Figure 7B).

Further, gender based analysis of $CD4^+CD25^{low}FoxP3^-$ Teff cells did not show significant difference in Teff% between male and female patients (p=0.996) (Figure 7C). Interestingly, when $CD4^+CD25^{low}FoxP3^-$ Teff cells were monitored in different age of onset groups of patients, patients with the age group 1-20 yrs (10.79 ± 0.6943) showed significant difference in Teff% as compared to the age groups 21-40 yrs (14.62 ± 1.275), 41-60 yrs (14.51 ± 1.891) and 61-80 yrs (15.05 ± 2.481) (p=0.006, p=0.032 and p=0.044 respectively) (Figure 7D).

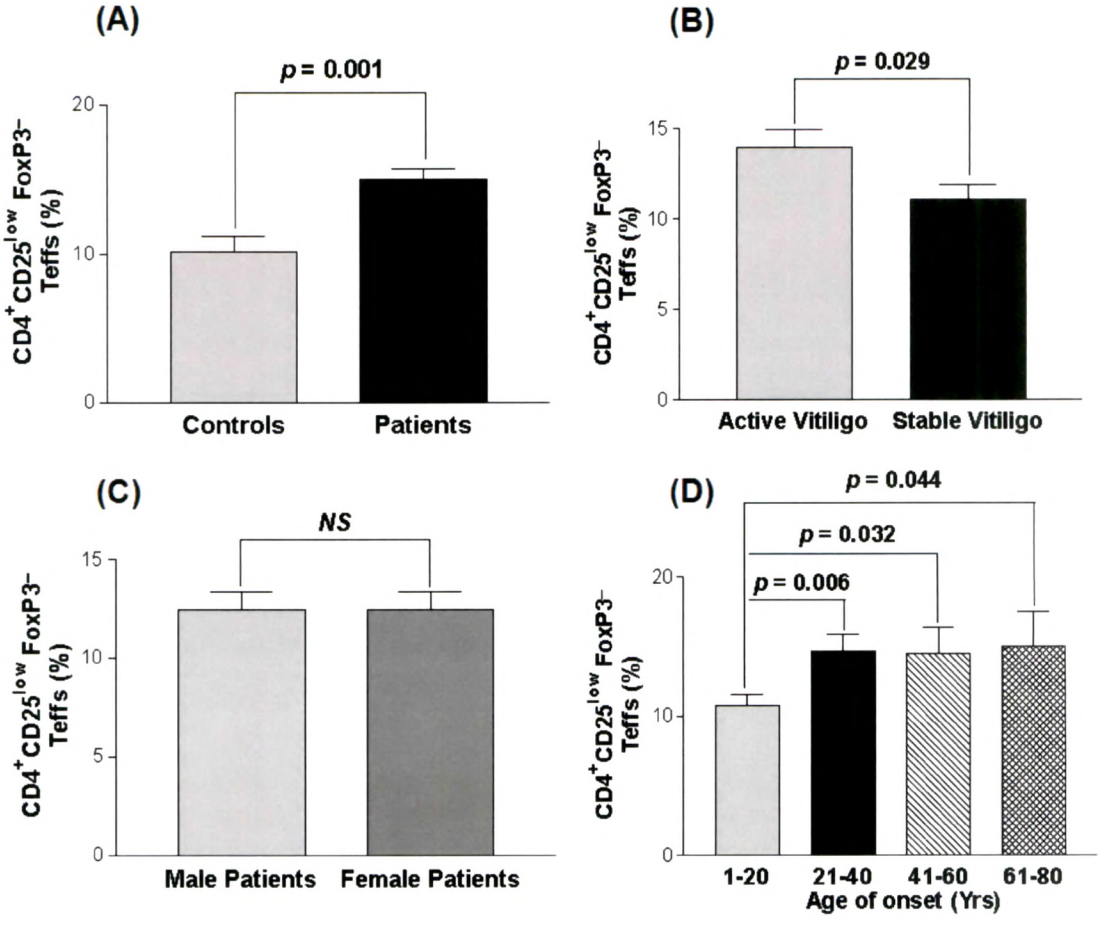


Figure 7. T-effector ($CD4^+CD25^{low}FoxP3^-$) cells in vitiligo patients and controls:

(A) $CD4^+CD25^{low}FoxP3^-$ Teff cells in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (10.16 ± 1.031 vs 14.98 ± 0.7445; p=0.001).

(B) $CD4^+CD25^{low}FoxP3^-$ Teff cells in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (13.96 ± 1.010 vs 11.09 ± 0.8013; p=0.029).

(C) $CD4^+CD25^{low}FoxP3^-$ Teff cells in male (n = 39) and female patients (n = 43) with vitiligo. Values are given as mean ± SEM: male vs female (12.45 ± 0.9285 vs 12.44 ± 0.9595; p=0.996) (NS=non-significant).

(D) $CD4^+CD25^{low}FoxP3^-$ Teff cells in different age of onset groups of patients (1-20 yrs, n = 40; 21-40 yrs, n=20; 41-60 yrs, n=16, 61-80 yrs, n=6). Values are given as mean ± SEM: 1-20 yrs vs 21-40, 41-60, 61-80 yrs (10.79 ± 0.6943 vs 14.62 ± 1.275, 14.51 ± 1.891, 15.05 ± 2.481; p=0.006, p=0.032 and p=0.044 respectively).

8.3.2.4 Ratio of Tregs to Teffector (Tregs/Teffs) cells in vitiligo patients

The Tregs/Teffs ratio (0.5420 ± 0.02668) was significantly lower in vitiligo patients than that of unaffected controls (0.6879 ± 0.07651) (p=0.025) (Figure 8A). Analysis based on the stage of progression of vitiligo suggested that ratio of Tregs/Teffs was significantly decreased in the 56 patients with active cases (0.5124 ± 0.03684) compared to 29 patients with stable vitiligo (0.7086 ± 0.05222) (p=0.003) (Figure 8B).

Further, gender based analysis of Tregs/Teffs ratio did not show significant difference between male and female patients (p=0.559) (Figure 8C). Also, when Tregs/Teffs ratio was analyzed in different age of onset groups of patients, patients with the age group 1-20 yrs did not show significant difference as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.741, p=0.874 and p=0.387 respectively) (Figure 8D).

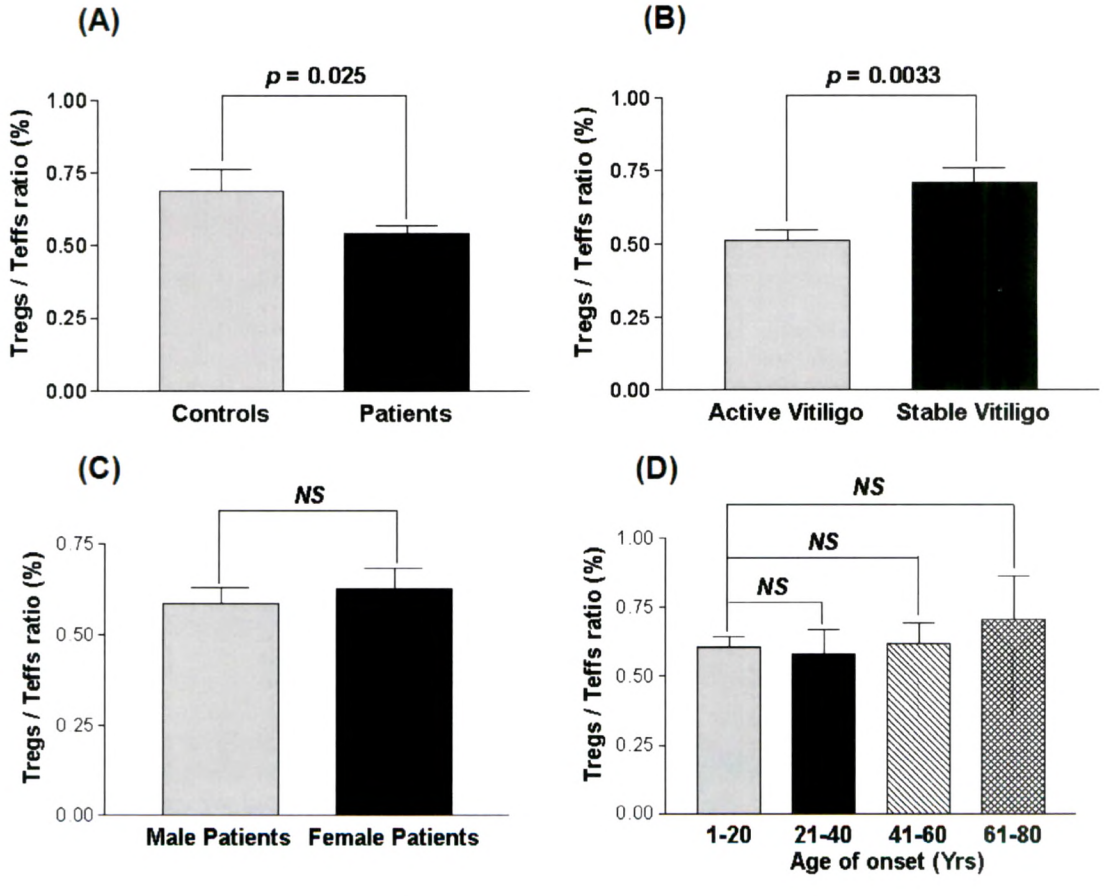


Figure 8. Ratio of Tregs to Teffector (Tregs/Teffs) cells in vitiligo patients and controls:

(A) Tregs/Teffs ratio in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (0.6879 ± 0.07651 vs 0.5420 ± 0.02668; p=0.025).

(B) Tregs/Teffs ratio in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (0.5124 ± 0.03684 vs 0.7086 ± 0.05222; p=0.003).

(C) Tregs/Teffs ratio in male (n = 39) and female patients (n = 43) with vitiligo. Values are given as mean ± SEM: male vs female (0.5880 ± 0.04181 vs 0.6286 ± 0.05439; p=0.559); (NS=non-significant).

(D) Tregs/Teffs ratio in different age of onset groups of patients (1-20 yrs, n = 40; 21-40 yrs, n=20; 41-60 yrs, n=16, 61-80 yrs, n=6). Values are given as mean ± SEM: 1-20 yrs vs 21-40, 41-60, 61-80 yrs (0.6071 ± 0.03855 vs 0.5794 ± 0.08790, 0.6206 ± 0.07374, 0.7080 ± 0.1549; p=0.741, p=0.874 and p=0.387 respectively); (NS=non-significant).

8.4 DISCUSSION

T cells are more prevalent in vitiligo perilesional skin than in surrounding non-lesional or lesional skin. An occasional T cell remains in lesional skin but most of the infiltrate appears to migrate with the depigmenting epidermal border. Among the T cells, the infiltrate consists mainly of $CD8^+$ but also of $CD4^+$ T cells (Le Poole *et al.*, 1996). Since cytotoxic T cells are especially prevalent and colocalize with remaining melanocytes, it was postulated that these T cells are actively cytolytic towards remaining melanocytes (Wankowicz-Kalinska *et al.*, 2003). The involvement of T cells in vitiligo is further supported by the observation that depigmentation is accompanied by the expression of type 1 cytokines (Le Poole *et al.*, 2004). Moreover, studies have previously demonstrated that melanocytes are capable of phagocytosis and can process and present antigens in the context of MHC class II to $CD4^+$ proliferative and cytotoxic T cells (Le Poole *et al.*, 1993a,b).

Association of Major Histocompatibility Complex (MHC) alleles with vitiligo gains importance because of the antigen-presenting function of the MHC. Our recent study suggests a consistent increase of *A*33:01*, *B*44:03*, and *DRB1*07:01*, implicating these alleles as possible markers of vitiligo in North India and Gujarat These data apparently suggest auto reactive CD4+ T-helper cells to be restricted by HLA-

*DRB1*07:01* and the auto-reactive CD8+ cytotoxic T cells by *HLA-A*33:01*, *A*02:01*, *B*44:03*, and *B*57:01* in the Indian populations studied (Singh *et al.*, 2012). In another recent study, we identified the three most significant class II region SNPs: rs3096691 (just upstream of *NOTCH4*), rs3129859 (just upstream of HLA-DRA), and rs482044 (between *HLA-DRB1* and *HLA-DQA1*) (unpublished data) associated with generalized vitiligo suggesting an important link between vitiligo and immune system.

Currently, studies presenting the reciprocal relationship between $CD4^+CD25^+Foxp3^+$ Tregs and $CD8^+$ in GV progression are lacking. $CD8^+$ Tcell-mediated tissue damage has been demonstrated in common organ-specific autoimmune diseases, such as type I diabetes and multiple sclerosis, and a role for $CD8^+$ T cells has been postulated in the pathogenesis of GV. Previous studies have largely focused on melanocyte-specific cytotoxic T lymphocytes (CTLs) and identified their pivotal role in inducing melanocyte destruction (Ogg *et al.*, 1998, Palermo *et al.*, 2001, Lang *et al.*, 2001). Several reports have shown that increase in globally activated $CD8^+$ CTLs correlate with disease activity in various autoimmune disorders (Blanco *et al.*, 2005; Giovanni *et al.*, 2011). Moreover, in progressive disease, the $CD4^+/CD8^+$ ratio is decreased among skin-infiltrating T cells and $CD8^+$ T cells isolated from vitiligo skin are cytotoxic to melanocytes (Wankowicz-Kalinska *et al.*, 2003). Recently, Lili *et al.* (2012) have demonstrated significantly higher number of circulating $CD8^+$ T among $CD3^+$ T cells in progressive GV.

A decrease in $CD4^+$ T-cell population and an increase in $CD8^+$ cells have also been observed in vitiligo cases by Grimes *et al.* (1986) and Halder *et al.* (1986). The present study also showed decreased $CD4^+/CD8^+$ ratio in vitiligo patients as compared to controls, indicating the prevalence of $CD8^+$ cells in patients. In addition, the peripheral $CD8^+$ cells were prevalent in active cases of vitiligo as compared to stable cases; however, there was no effect of age of onset and gender biasness on $CD4^+/CD8^+$ ratio in patients suggesting that the decreased $CD4^+/CD8^+$ ratio mainly affects the progression of the disease.

Recently, decreased ratio of $CD4^+/CD8^+$ and increase in $CD8^+$ cells was demonstrated in vitiligo patients from India suggested important role of $CD8^+$ cells in the T cell mediated melanocyte death (Nigam *et al.*, 2011). However, the study could not

achieve stastical significance for the $CD4^+/CD8^+$ ratio in active and stable cases of vitiligo. In contrast, Pichler *et al.* (2009) reported increased $CD4^+/CD8^+$ ratio in vitiligo patients; however 40% of their patients had associated autoimmune thyroiditis which might have influenced their reults. Thus far, the immune mechanisms underlying the induction and activation of autoreactive $CD8^+$ CTLs and the loss of tolerance to auto-antigens are not clear. MART-1 and gp100 were first identified as the target antigens for T cells infiltrating melanoma tumors suggesting why the autoimmune response to melanocytes effectively eliminates melanocytes from vitiligo skin whereas T cells infiltrating melanomas fail to clear the tumor. An abundance of Tregs in tumor tissues is thought to be the root cause of failing attempts to boost anti-tumor immunity by powerful vaccines, and inclusion of FoxP3 as a target antigen in vaccines was shown to boost anti-tumor immunity (Loddenkemper *et al.*, 2009; Nair *et al.*, 2007). It has been proposed that failure to suppress an ongoing immune response to self antigens may contribute to progressive depigmentation of the skin in vitiligo patients (Das *et al.*, 2001).

Natural Treg cells play a key role in maintaining peripheral tolerance *in vivo* through the active suppression of self-reactive T cell activation and expansion (Klarquist *et al.*, 2010). Dysfunction or deficiency of Tregs has been reported in several autoimmune diseases, both systemic (Antiga *et al.*, 2010; Miyara *et al.*, 2005) and organ-specific (Chi *et al.*, 2008).

The most intriguing Tregs are those showing $CD4^+CD25^{hi}FoxP3^+$ phenotype. $CD4^+CD25^{hi}$ cells constitute 4-10% whereas $CD4^+CD25^{low}$ cells comprise 22-23% of peripheral blood lymphocytes (Sasaki *et al.*, 2004; Sugiyama *et al.*, 2005). Several other markers have also been described for Tregs, such as surface-bound latency-associated peptide/transforming growth factor β1 (LAP/TGF-β1), glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), neuropilin-1, galectin-1, and lymphocyte activation gene-3 (LAG-3). Furthermore, toll like receptors (TLRs), mainly expressed on antigen-presenting cells (APC) as a bridge linking innate and adaptive immunity, also exist on Tregs (Zhang *et al.*, 2008). These Tregs play a major role in tuning inflammatory response to infections, in guarding against autoimmunity and play a role in tumor progression (Afzali *et al.*, 2007). It has been proposed from murine

melanoma studies, that depletion of Tregs would activate antimelanoma cytotoxic T-cells that destroy melanoma and generate vitiligo as a side effect (Zhang *et al.*, 2007). Regulatory T cells are known to inhibit autoreactivity, explaining why autoreactive T cells are present in the circulation in the absence of autoimmune symptoms (Baecher-Allen and Hafler, 2006). While the mechanism of action for Tregs is still not fully understood, TGF-β and IL-10 contribute to Treg mediated immunosuppression. TGF-β is important for imposing a regulatory phenotype to the Treg subset and regulatory activity is dependent on cell-cell contact (Bala and Moudgil, 2006; Joetham *et al.*, 2007; Zhu and Paul, 2008).

The level of $CD4^+CD25^{hi}$ $FoxP3^+$ Tregs has been variably reported in different autoimmune diseases. A decrease in the proportion of circulating Tregs among other $CD4^+$ T-cells is reported in active cases of systemic lupus erythematosus. In contrast, the proportions of Tregs in primary Sjögren syndrome, inflammatory myopathy (Miyara *et al.*, 2005), type I diabetes mellitus (Lindley *et al.*, 2005) and multiple sclerosis (Viglietta *et al.*, 2004) do not significantly differ from controls values. As for autoimmune skin diseases, Tregs were normal in number in serum of psoriatic patients (Sugiyama *et al.*, 2005) and were decreased in pemphigus vulgaris (Sugiyama *et al.*, 2007).

Currently, very little is known about the function of Treg cells in GV patients. To date, a few studies have investigated Treg numbers or function in GV patients. In this study, we show that GV patients exhibit a decreased prevalence of circulating Tregs; however a recent study has demonstrated that the percentage of peripheral $CD4^+CD25^+FoxP3^+$Tregs remains unaltered in generalized vitiligo (Zhou *et al.*, 2012). Our results are in concordance with those of Lili *et al.*, 2012 indicating that reduced numbers and impaired function of natural Tregs fail to control the widespread activation of $CD8^+$CTLs, which leads to the destruction of melanocytes in GV. Nevertheless, Abdallah and Saad evaluated the level of $CD4^+CD25^{hi}FoxP3^+$ Tregs in generalized vitiligo and their relation to effector T-cells (Teffs) demonstrating the elevation of Tregs and Teffs (Abdallah and Saad, 2009). Klarquist *et al.*, revealed a defect in Treg cell homing to the skin, based on the finding of drastically reduced Treg numbers in vitiligo skin without any systemic drop in their abundance or activity (Klarquist *et al.*, 2010). However, a recent report identified increased numbers of

Tregs in perilesional skin despite a functional defect of circulating Tregs in progressive vitiligo (Ben Ahmed *et al.,* 2011). Moreover, in the present study FoxP3 expression in the $CD4^{+}CD25^{hi}$ Treg cells was found to be significantly decreased in cases of vitiligo as compared to controls suggesting that the Tregs may have functional defects which can affect their suppressive activity. Patients with an autoimmune disease such as systemic lupus erythematosus have a relative dysfunction of FoxP3 positive cells (do-Sánchez *et al.,* 2006). The dysfunction of FoxP3 may be due to mutations e.g. in the X-linked IPEX syndrome (immunodysregulation polyendocrinopathy and enteropathy X-linked) mutations were in the forkhead domain of FoxP3, indicating that the mutations may disrupt its critical DNA interactions (Bennett *et al.,* 2001). Recently, Lili *et al.* (2012) and Ben Ahmed *et al.* (2011) have demonstrated that the suppressive effects of peripheral T regulatory cells in progressive GV cases were significantly reduced, demonstrating an impairment in their ability to inhibit the proliferation and cytokine production of stimulated autologous $CD8^{+}$ T cells. Gerold *et al.,* (2011) also showed decreased sCTLA-4 levels in type-1 diabetic condition and suggested that lower sCTLA-4 expression may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. Our findings also suggested decreased levels of both sCTLA-4 and flCTLA-4 suggestive of the disturbance in the suppressive capacity of Tregs in vitiligo patients (Dwivedi *et al.,* 2011). Thus, a functional defect in Tregs might be involved in the pathogenesis of vitiligo.

Interestingly, our results suggest that female patients showed significant low expression of FoxP3 in $CD4^{+}CD25^{h}$i Treg cells as compared to male patients suggestive of the fact that females have increased susceptibility towards vitiligo as compared to males, implicating sex bias in the development of autoimmunity (Whitacre, 2001; Panchanathan and Choubey, 2012; Afshan *et al.,* 2012). However, we did not find the significant difference in Treg% between female and male patients with vitiligo.

In addition, we found decreased Treg cells in early age group of patients (1-20 yrs) as compared to the late onset groups which suggests the crucial role of Treg cells in early phase of the disease. Moreover, the Teff cells were found to be increased in vitiligo patients as compared to controls suggesting the effect of reduced number

and/dusfunction of Treg cells in patients. Also, active cases of vitiligo showed significant increase in Teff cells which further signifies the importance of Tregs in progression of the disease. Previous studies showed increased Teff cells in progressing vitligo (Abdallah and Saad, 2009; Lili *et al.,* 2012). Furthermore, the Treg/Teff cells ratio was found to be decreased in vitligo patients as compared to controls suggesting the increased Teff cells in patients. The Treg/Teff cells ratio was significantly decreased in active cases of vitligo as compared to stable cases, indicating the role of Teff cells in progression of the disease.

It is possible that high affinity T cells previously escaped clonal deletion in skin-draining lymph nodes and were inadvertently allowed to enter the circulation, emigrating to the skin to inflict damage to the melanocyte population (Palermo *et al.,* 2005). An ongoing autoimmune response may be allowed to further develop and mature in the absence of functional T regulatory cells (Tregs) (Dejaco *et al.,* 2006). These cells actively mediate suppression of the immune system generally by secreting IL-10 and TGF-β to prevent autoimmunity (Dejaco *et al.,* 2006). Our recent data suggested that IL-10 expression is decreased in vitiligo patients as compared to controls indicating that decreased levels of IL-10 may affect the supressive capacity of Tregs. The present study showing a decrease in the number of Tregs in vitiligo skin indicates that this may apply to vitiligo as well. In the absence of regulatory T cells, cytotoxic T cells with increasing affinity for their targets enter the skin and continuously proliferate and migrate towards novel target cells, causing depigmentation.

In conclusion, our study indicates that an imbalance of $CD4^+/CD8^+$ ratio and natural Tregs in frequency and function might be involved in the T-cell mediated pathogenesis of vitiligo and its progression. The reduced levels of natural Treg cell population might lead to a global expansion and widespread activation of the $CD8^+$ population, which could result in the destruction of melanocytes in vitiligo patients.

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CONCLUDING REMARKS

Vitiligo is a common skin disorder characterized by the acquired loss of constitutional pigmentation manifesting as white macules and patches caused by loss of functional epidermal melanocytes. Despite tremendous progress in molecular biology and genetics, there is still no universally accepted hypothesis. It could well be that vitiligo represents a 'syndrome' rather than a disease, with numerous different but not mutually exclusive pathways leading to melanocyte failure or disappearance. The exact etiology of vitiligo remains obscure, but autoimmunity has been strongly implicated in the development of disease, especially in generalized vitiligo. However, genetic factors probably determine which particular pathway predominates in a specific patient. Several immunogenetic factors predispose patients to autoimmune diseases and therefore the present study addresses the role of a few candidate genes involved in autoimmune pathogenesis of vitiligo.

Vitiligo is widely considered as an autoimmune disease, with involvement of humoral and cellular components of immune system. The evidences in support to this hypothesis are: association with other autoimmune disorders; chronic relapsing and remitting course typical of autoimmune disorders; possible response to immunosuppressive therapies; circulating anti-melanocyte antibodies; T-cell infiltrates in perilesional skin; anti-melanocyte cytotoxic T-cells in the skin and circulation and proinflammatory cytokine patterns of a Th-1 type response. Autoimmunity might not be the triggering event in vitiligo, but it could function instead as a promoter of disease progression and chronicity.

The present study evaluates the autoimmune hypothesis of vitiligo pathogenesis by addressing the levels of antimelanocyte antibodies and monitoring the T-cell subsets in vitiligo patients of Gujarat population. We found significantly higher levels of antimelanocyte antibodies in the circulation of patients and these antibodies correlate with disease activity and extent. Active vitiligo patients exhibited increased levels of antimelanocyte antibodies as compared to stable vitiligo patients. Moreover, patients with early age of onset had significantly higher levels of antimelanocyte antibodies suggesting the role of antimelanocyte antibodies in early onset of the disease. Gender based analysis for antimelanocyte antibodies revealed that female patients had higher levels of antimelanocyte antibodies as compared to male patients suggesting that

females are more susceptible towards autoimmune diseases. Targets of these antibodies include a variety of melanocyte and melanosomal antigens. Antibodies might trigger vitiligo as a primary event, but they could also arise secondary to melanocyte damage or serve to perpetuate the disease. Studies suggested that whatever may be their role in vitiligo pathogenesis; these antibodies have the capacity to injure melanocytes *in vivo* and *in vitro*.

The high frequencies of melanocyte-reactive cytotoxic T cells in the peripheral blood of vitiligo patients, peri-lesional T-cell infiltration and melanocyte loss *in situ* suggest the important role of cellular autoimmunity in the pathogenesis of this disease. In most vitiligo patients the balance of cytotoxic/suppressor and helper/inducer T-cells in peripheral blood is disturbed which might lead to predominance of distinct T-cell subtypes. Moreover, in progression of disease, the $CD4^+/CD8^+$ ratio is decreased among skin-infiltrating T cells. $CD8^+$ T cells isolated from vitiligo skin are cytotoxic to melanocytes. Our results showed that $CD4^+/CD8^+$ ratio was significantly lowered in generalized vitiligo patients as compared to controls. The $CD4^+/CD8^+$ ratio was further lowered, as $CD8^+$ T-cell count was significantly higher in patients with active vitiligo as compared to stable vitiligo suggesting the role of $CD8^+$ T-cells in progression of the disease.

Association of Major Histocompatibility Complex (MHC) alleles with vitiligo gains importance because of the antigen-presenting function of the MHC. Our recent study suggests a consistent increase of A*33:01, B*44:03, and DRB1*07:01, implicating these alleles as possible markers of vitiligo in North India and Gujarat These data apparently suggest auto reactive CD4+ T-helper cells to be restricted by HLA-DRB1*07:01 and the auto-reactive CD8+ cytotoxic T cells by HLA-A*33:01, A*02:01, B*44:03, and B*57:01 in the Indian populations studied (Singh et al., 2012). In another recent study, we identified the three most significant class II region SNPs: rs3096691 (just upstream of NOTCH4), rs3129859 (just upstream of HLA-DRA), and rs482044 (between HLA-DRB1 and HLA-DQA1) (unpublished data) associated with generalized vitiligo suggesting an important link between vitiligo and immune system.

Natural Treg cells play a key role in maintaining peripheral tolerance *in vivo* through the active suppression of self-reactive T cell activation and expansion, and dysfunction or deficiency of Tregs may result into autoimmune diseases including vitiligo. The most intriguing Tregs are those showing $CD4^+CD25^{hi}FoxP3^+$. However, very little is known about the function of Treg cells in generalized vitiligo (GV). The level of $CD4^+CD25^{hi}$ $FoxP3^+$ Tregs has been variably reported in different studies. In this study, we have found that GV patients exhibit a decreased prevalence of circulating Tregs and hence an increase in Teff cell population was observed in these patients which might be responsible for melanocyte destruction. Moreover, the FoxP3 expression in the $CD4^+CD25^{hi}$ Treg cells was found to be significantly decreased in cases of vitiligo as compared to controls suggesting that the Tregs may have functional defects which can affect their suppressive activity. In addition, our recent study suggests decreased level of IL10 in vitiligo patients, which is secreted by Tregs for supression of activated T-cells. We found that female patients showed significant low expression of FoxP3 in $CD4^+CD25^{hi}$ Treg cells as compared to male patients suggesting that females have increased susceptibility towards autoimmunity. Thus, a functional defect in Tregs might be involved in the pathogenesis of vitiligo. Early age group of patients (1-20 yrs) showed lower levels of Treg cells as compared to the late onset groups which suggests the crucial role of Treg cells in early onset of the disease.

Also, active cases of vitiligo showed significant increase in Teff cells which further signifies the importance of Tregs in progression of the disease. Thus, the present study indicates that an imbalance of $CD4^+/CD8^+$ ratio and natural Tregs in frequency and function might be involved in the T-cell mediated pathogenesis of vitiligo and its progression. The reduced levels of natural Treg cell population might lead to a global expansion and widespread activation of the $CD8^+$ population, which could result in the destruction of melanocytes in vitiligo patients.

CTLA4 is an important molecule in the down-regulation of T-cell activation and it is critical for the activity of regulatory T cells. The present study showed a significant decrease in *sCTLA4* and *flCTLA4* expression suggesting that lower levels of *s*CTLA4 and *fl*CTLA4 may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. We found that expression of *CTLA4* in vitiligo patients was

modulated by CT60AG dimorphism (rs3087243). The CT60A/G polymorphism was significantly associated with susceptibility to vitiligo in Gujarat population. Thus, our study suggests that the dysregulated *CTLA4* expression in vitiligo patients could result, at least in part, due to variations at the genetic level. For the first time, we showed that the 3' UTR CT60A/G polymorphism of the *CTLA4* gene influences both full length and soluble *CTLA4* mRNA levels in vitiligo patients, and thus this genotype-phenotype correlation of *CTLA4* supports the autoimmune hypothesis of vitiligo pathogenesis (Dwivedi *et al.*, 2011).

The epidermal imbalance in skin of vitiligo patients suggests the role of cytokines in vitiligo skin autoimmunity. Cytokine studies of peripheral blood and skin in patients with vitiligo have yielded variable results, but with a trend to proinflammatory T-cell patterns. Studies suggested that topical tacrolimus, used successfully to treat vitiligo, increases tissue IL-10, which is an immunosuppressive Th-2 cytokine. This suggests that vitiligo might be a Th-1 type of autoimmune disease. IFNγ is one of the important Th-1 cytokine involved in the activation of antigen presenting cells (APCs). It promotes Th-1 differentiation by up regulating the transcription factor NF-kB and inhibits the development of Th-2 cells thereby playing role in maintaing the Th-1/Th-2 ratio which predominantly decides the development of autoimmunity. Our results suggest increased mRNA and protein levesl of IFNγ in vitiligo patients. The genetic polymorphisms of *IFNG* intron 1 +874A/T (rs2430561) with CA repeats (rs3138557) is reported to modulate its expression through NF-kB. We found significant association of high producer 12 CA repeats with vitiligo. Moreover, the intron 1 'T' allele as well as 12 CA repeat were correlated with higher mRNA and serum IFNγ levels in vitiligo patients. The study suggests that *IFNG* CA microsatellite but not +874 A/T may be a genetic risk factor for generalized vitiligo in Gujarat population; however, +874T allele plays a major role in increased expression of *IFNG* mRNA and protein levels which can affect the onset and progression of disease.

IFNγ stimulates the expression of intercellular adhesion molecule 1 (*ICAM1*), which is important for activating T cells and recruiting leukocytes. ICAM1 protein levels are upregulated in vitiligo skin and in melanocytes from perilesional vitiligo skin. The present study also showed increased expression of *ICAM1* in vitiligo patients

suggesting that increased IFNγ levels might be responsible for increased *ICAM1* expression in vitiligo patients. It has been reoprted that increased expression of this adhesion molecule on the melanocytes enhances T cell -melanocyte attachment in the skin and may lead to the destruction of melanocytes. Moreover, the *ICAM1* expression was increased in active cases of vitiligo as compared to stable vitiligo suggesting its role in progression of the disease. The *ICAM1* expression was increased with early age of onset of the disease further implicating the important role of *ICAM1* in early phase of the disease. Also, female patients showed an increased expression of *ICAM1* as compared to male patients suggesting that females have more susceptibility towards vitiligo. Thus, the study suggests that increased IFNγ levels in patients may lead to increased *ICAM1* expression which is probably an important link between cytokines and T cells involved in vitiligo pathogenesis.

Recent genome-wide association studies (GWAS) revealed a highly significant association of familial cases of generalized vitiligo with polymorphic variants of the gene encoding *NALP1* - a key regulator of the innate immune system. Immunohistochemical studies have found high *NALP1* expression in epidermis within Langerhans cells and T cells suggesting involvement of *NALP1* in skin autoimmunity. *NALP1* plays a crucial role in the assembly of the apoptosome thereby it is involved in inflammation and apoptosis. *NALP1* may also contribute to modulate the response of cells towards proinflammatory stimuli like IL1β, IFNγ and TNFα. Overexpression of this gene induces apoptosis in cells. In the present study we found increased expression of *NALP1* in vitiligo patients. The promoter (rs2670660) and intronic (rs6502867) variants of *NALP1* were significantly associated with vitiligo susceptibility. The genotype-phenotype correlation of these SNPs revealed that susceptible genotypes confer increased expression of *NALP1* in vitiligo thereby implicating its crucial role in vitiligo pathogenesis. The study also emphasizes the influence of *NALP1* on the disease progression, onset of the disease and gender biasness for developing vitiligo.

MYG1 is a ubiquitous nucleo-mitochondrial protein involved in early developmental processes. Studies showed that siRNA mediated knockdown of *MYG1* resulted in the altered levels of transcripts encoding transcription factors involved in development

and growth as well as immune-related processes. *MYG1* promoter and structural polymorphisms have a functional impact on the regulation of *MYG1* gene. Elevated expression of *MYG1* mRNA in both uninvolved and involved skin of vitiligo patients has been reported. The present study showed that systemic expression of *MYG1* transcript is elevated in vitiligo patients as compared to controls. Also, patients with active vitiligo and early age of onset had elevated levels of *MYG1* transcript which further suggested the important role of MYG1 in progression of the disease. The *MYG1* mRNA levels in patients were also correlated with *MYG1* promoter polymorphism -119C/G (rs1465073). Thus, our results confirm that -119C/G polymorphism influences *MYG1* transcript levels and minor -119G allele is the risk-allele for the development of vitiligo. The study also emphasizes the influence of MYG1 on the disease progression, onset of the disease and gender biasness for developing vitiligo.

MBL2 plays an important role in innate immunity. It helps in the clearance of apoptotic cells and in complement activation. Genetic variability due to structural and promoter polymorphisms in *MBL2* gene has been reported to be associated with increased risk for several autoimmune diseases including vitiligo. *MBL2* exon variants (rs5030737, rs1800450, rs1800451) cause structural changes in the collagen like domain of MBL2, whereas *MBL2* promoter variants (rs7096206) lead to its low plasma levels. However, the present study could not find association of these variants with vitiligo susceptibility (Dwivedi *et al.,* 2009).

Moreover, *PTPN22* which is a T-cell down regulator gene has a geneitc variant (1858C/T) (rs2476601) which leads to alteration in T-cell suppressive capacity; however, this SNP was not associated with vitiligo susceptibility in Gujarat population (Laddha *et al.,* 2008). In addition, *ACE* gene which catalyzes the conversion of angiotensin I to angiotensin II is a potent mediator of oxidative stress and stimulates the release of cytokines and the expression of leukocyte adhesion molecules that mediate vessel wall inflammation. Inflammatory cells release ACE that generates angiotensin II which in turn can induce cytokines such as TNFα and IFNγ. Our recent studies found increased transcript and protein levels of TNFα in vitiligo patients. These increased cytokines may modulate the microenvironment which in turn can signal for apoptosis of melanocytes as observed in vitiligo patients. The *ACE* I/D polymorphism (AF118569) is reported to alter the ACE levels;

however, the present study could not find significant association of this polymorphism (Dwivedi *et al.,* 2007). Thus, our study proposes that the genetic variants in *MBL2, PTPN22* and *ACE* genes may not be a genetic risk factor for generalized vitiligo susceptibility in Gujarat population.

Overall, our studies find genetic predisposition in immune regulator genes including *CTLA4, IFNG, NALP1* and *MYG1* which can modulate the immune response towards melanocytes. Moreover, the presence of increased antimelanocyte antibodies and the imbalace of T-cell subsets along with their functional defects might result in melanocyte destruction in vitiligo patients. However, a single dominant pathway appears unlikely to account for all cases of melanocyte loss in vitiligo and apparently, a complex interaction of genetic, environmental, biochemical and immunological events is likely to generate a permissive milieu. It is most likely that loss of melanocytes in vitiligo occurs through a combination of several pathogenic mechanisms at molecular and cellular levels that act in concert (Figure 1).

Further studies on melanocyte antibodies and target antigens along with possible trigerring factors for generation of autoimmunity in vitiligo patients might refine the diagnostic and prognostic testing of vitiligo and also reveals putative T-cell targets which add to the therapeutic armamentarium.

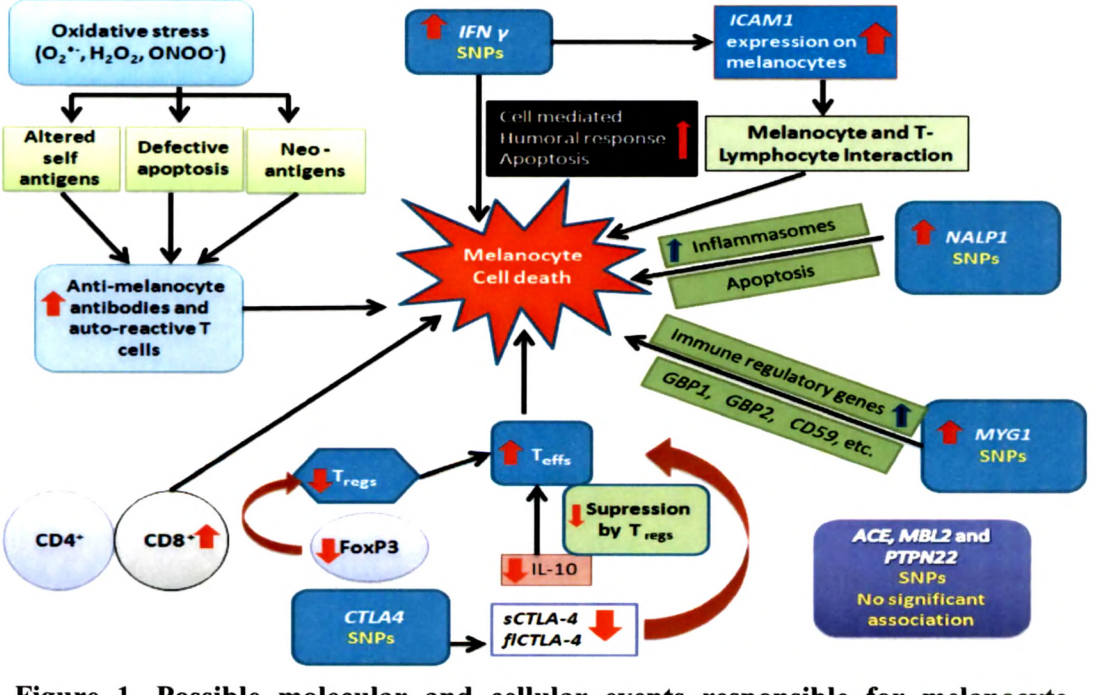

Figure 1. Possible molecular and cellular events responsible for melanocyte destruction in autoimmune pathogenesis of vitiligo:

Melanocyte cell death in vitiligo is a complex process triggered by several different pathways. One of the normal pathways is disturbed i.e., the regulation of T-cells as $CD8^+$ T-cell counts are increased in patients resulting in melanocyte death. Tregs which are important in suppression of activated T-cells are decreased in vitiligo patients. The lowered Treg levels may be due to decreased FoxP3 expression thereby increasing the levels of Teff cells. Moreover, increased Teff cells may be due to low *IL10* expression and thereby conferring low suppressive capacity of Tregs as observed in vitiligo patients. Another important regulator of T-cells is *CTLA4*. Both *sCTLA4* and *flCTLA4* levels were decreased in vitiligo patients and correlated with the presence of SNPs in *CTLA4*. Decreased *CTLA4* expression increases the Teff cells resulting in melanocyte death. A Th-1 cytokine IFNγ is increased in vitilgio patients and its expression is correlated with its polymorphisms. IFNγ is a paracrine inhibitor of melanocyte and hence can result in melanocyte destruction via enhancing humoral, cell mediated immunity and apoptosis. In turn IFNγ induces the expression of *ICAM1* on melanocytes. Increased *ICAM1* expression as observed in vitiligo enhances the melanocyte and T-lymphocyte interaction and thereby leading to melanocyte death. Expression of *NALP1* is increased in vitiligo patients and correlated with the presence of SNPs. Increased *NALP1* expression results in increased inflammasomes formation leading to apoptosis of melanocytes. MYG1, a stress induced molecule is increased in vitiligo patients. Increased *MYG1* levels result in overexpression of several immune regulator genes thereby affecting melanocyte death.

High levels of ROS and RNS are observed in vitiligo patients, which can directly cause melanocyte death. Moreover, the free radicals can react with cellular constituents resulting in altered antigenicity of the self antigens, formation of neo-antigens and cause defects in the apoptotic machinery of the cell, ultimately provoking humoral and cell mediated immunity by producing antimelanocyte antibodies and auto-reactive T-cells leading to melanocyte destruction. Certain other molecules like ACE, PTPN22 and MBL2 with their mutant proteins may directly or indirectly participate in melanocyte destruction.

Vitiligo Clinical Proforma

Department of Biochemistry, The Maharaja Sayajirao University of Baroda,
Faculty of Science, Vadodara, Gujarat - 390 002

Dr. _____ Date: _____
Name: _____ Age: _____ Sex: _____

Address_____
_____

Marital status: Married/Single Religion: _____

Occupation: _____ Income: _____

Education: _____ Native Place: _____

History of illness

1. Age of onset: _____
2. Site of onset: _____
3. Duration: _____
4. Lesions: Number_____ Size: _____ Shape_____
5. Condition of hair: no/ black/ gray
6. Any associated symptoms: Itching/ burning/ pain
7. Mode of spread: Static/ growing/ receding
8. Use of any drugs before onset of illness
   _____
   _____
   _____
   _____
9. Aggravating factors: occupational/ hobbies/ trauma/ drug/ work/sunlight/ emotional factors/ menstruation/ pregnancy/ food/ cosmetics/ chemicals/ any other:_____
10. What does the patient associate it with as cause_____
    _____
11. Treatment: yes/ no Regular/ Irregular
12. Recovery: Some/ good/ poor/ no response
13. Sudden repigmentation: yes/ no
14. Local sensitivity (photo):
    _____

Appendix I

15. Associated diseases:

_____

_____

16. Family history
    A. I[st] degree relatives: father/mother/sister/brother/daughter/son
    B. 2[nd] degree relatives: paternal grandmother/paternal grandfather/ maternal grandmother/maternal grandfather/maternal or paternal uncles or aunts
    C. 3[rd] degree relatives: cousins/nephews/nieces

17. Personal history
    Diet: veg/ nonveg/ ovoveg/ mixed

    Routine food: _____

18. Habits: smoking/ tobacco chewing/ alcoholism

19. Types of vitiligo
    a. Generalized/ Localized
    b. Unilateral/ Bilateral
    c. Symmetrical/ Symmetrical
    d. Universal
    e. Acrofacial
    f. Segmental
    g. Focal
    h. Liptip vitiligo
    i. Trichrome
    j. Quadrichrome

20. Treatment:

_____

_____

21. Koebners phenomenon: Yes/ No

The purpose of the study has been explained to me. I..... hereby agree to donate 5 ml of blood sample for the research purpose.

Signature of Patient

Date:

Appendix I

CONTROL CLINICAL PROFORMA

Department of Biochemistry

Faculty of Science

The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat- 390 002

Date: _____

Name: _____ Age: _____ Sex: _____

Address_____

Marital status: Married/Single Religion:

_____

Blood Group:

Occupation: _____ Income: _____

Education: _____ Native place: _____

1. Any Disease (including Vitiligo):

   _____

---

2. Personal history
   Diet: Veg/ nonveg/ ovoveg/ mixed

   Routine food: _____

3. Habits: Smoking/ tobacco chewing/ alcoholism

I, _____ have understood the aim of this study and willing to donate 5 ml blood sample for this purpose.

Signature

List of Publications

Imran M*, Laddha NC*, **Dwivedi M**, Mansuri MS, Singh J, Rani R, Gokhale RS, Sharma VK, Marfatia YS and Begum R (2012). Interleukin-4 genetic variants correlate with Its transcript and protein levels in vitiligo patients. ***Brit J Dermatol.*** 167: 314-323. (*Equal contribution)

Dwivedi M, Laddha NC, Imran M, Shah BJ and Begum R (2011). Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) in isolated vitiligo: a genotype-phenotype correlation. ***Pigment Cell Melanoma Res.*** 24: 737-740.

Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK, Gupta S, Laddha NC, **Dwivedi M**, Begum R, The Indian Genome Variation Consortium, Gokhale RS and Rani R (2012). HLA alleles and amino acid signatures of the peptide binding pockets of HLA molecules in Vitiligo. ***J Invest. Dermatol.*** 132: 124-134.

Dwivedi M, Gupta K, Gulla KC, Laddha NC, Hajela K and Begum R (2009). Lack of genetic association of promoter and structural variants of mannan-binding lectin (*MBL*-2) gene with susceptibility to generalized vitiligo. ***Brit J Dermatol.*** 161: 63-69.

Dwivedi M*, Laddha NC*, Shajil EM, Shah BJ and Begum R (2008). The *ACE* gene I/D polymorphism is not associated with generalized vitiligo susceptibility in Gujarat population. ***Pigment Cell Melanoma Res.*** 21: 407-408. (*Equal contribution)

Laddha NC, **Dwivedi M**, Shajil EM, Prajapati H, Marfatia YS and Begum R (2008). Association of *PTPN*22 1858C/T polymorphism with vitiligo susceptibility in Gujarat population. ***J Dermatol Sci.*** 49: 260-262.

Manuscripts under Communication

Dwivedi M, Laddha NC, Mansuri MS, Shah K, Begum R. Correlation of increased *MYG*1 expression and its promoter polymorphism with disease progression and higher susceptibility in vitiligo patients.

Laddha NC, **Dwivedi M**, Mansuri MS, Ansarullah, Ramachandran AV, Dalai S and Begum R. Vitiligo: interplay between oxidative stress and immune system.

Laddha NC, **Dwivedi M** and Begum R. Increased Tumor Necrosis Factor (*TNF*)-α and its promoter polymorphisms correlate with disease progression and higher susceptibility towards vitiligo.

Birlea SA, Ahmad FJ, Uddin RM, Ahmad S, Begum R, Laddha NC, **Dwivedi M**, Mansuri MS, Jin Y, Gowan K, Riccardi SL, Ben S, Fain PR and Spritz RA. Association of Generalized Vitiligo with HLA Class II Loci in Patients from the Indian Subcontinent.

Oral and Poster Presentations

Laddha NC, **Dwivedi M**, M. Imran, Shah K, Shah BJ and Begum R. "Role of TNF-α promoter polymorphisms in vitiligo susceptibility: a genotype-phenotype correlation." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M.S. University of Baroda, Vadodara.

Dwivedi M, Laddha NC, M. Imran, Shah K, Shah BJ and Begum R. "Role of interferon-γ polymorphisms in vitiligo susceptibility." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M. S. University of Baroda, Vadodara.

Mansuri MS, Laddha NC, **Dwivedi M**, Imran M, Agarwal N, Vasan K, Marfatia YS and Begum R. "Association of glutathione peroxidase 1 structural polymorphisms in relation to its activity and oxidative stress with vitiligo susceptibility." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M. S. University of Baroda, Vadodara.

Agarwal N, Laddha NC, **Dwivedi M**, Imran M, Mansuri MS , Patel HH , Shah AM , Marfatia YS and Begum R "Genetic polymorphisms of Neuropeptide-Y and Interleukin-1 beta may confer susceptibility to vitiligo." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M.S. University of Baroda, Vadodara.

Rani R, Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK, Gupta S, Laddha NC, **Dwivedi M**, Begum R, Indian Genome Variation Consortium, Gokhale RS. "Functional implications of MHC associations in vitiligo." **37th Annual Meeting of The American Society for Histocompatibility and Immunogenetics (ASHI),** Sheraton New Orleans, October 17-21, 2011.

Laddha NC, **Dwivedi M**, Imran M, Gani AR, Patel KJ, Sinh MK, Nateshan N, Parmar SS and Begum R. "Genetic polymorphisms in promoter region of TNF-α gene may confer susceptibility to vitiligo in Gujarat population." 4th Pan Arab

Human Genetics Conference on "Genomics of Human Diversity and Heritable Disorders" **Human Genome Meeting-2011**, 14th -17th March, 2011, Dubai.

Dwivedi M, Laddha NC, Imran M, Ichhaporia V and Begum R. "Association of Cytotoxic T Lypmhocyte Associated Antigen-4 (*CTLA*-4) 3' UTR CT60A/G and exon1 +49A/G single nucleotide polymorphisms with vitiligo susceptibility." 4th Pan Arab Human Genetics Conference on "Genomics of Human Diversity and Heritable Disorders" **Human Genome Meeting-2011**, 14th -17th March, 2011, Dubai. ****(Received the HUGO Young Scientist Travel Award)***.

Imran M, Laddha NC, **Dwivedi M**, Parmar CB, Khan F, Raimalani VM, Panchal VN and Begum R. "Genetic polymorphisms -590 C/T and intron 3 indel in interleukin 4 gene are in association with vitiligo in Gujarat population." HGM, 2011. 4th Pan Arab Human Genetics Conference on "Genomics of Human Diversity and Heritable Disorders" **Human Genome Meeting-2011**, 14th -17th March, 2011, Dubai.

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LETTER TO THE EDITOR

Association of *PTPN22* 1858C/T polymorphism with vitiligo susceptibility in Gujarat population

Sir,

Vitiligo is a depigmenting disorder resulting from the loss of functional melanocytes in the skin and affects 1–2% of the world population. Gujarat state has the highest prevalence, i.e., ~8.8%. Though vitiligo is extensively addressed in the past five decades, its etiology is still being debated [1]. Our biochemical studies have shown an impaired antioxidant defense mechanism in Gujarat vitiligo patients compared to controls [2,3]. Vitiligo is hypothesized to be of autoimmune origin [4]. Protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene C/T single nucleotide polymorphism at 1858 (rs2476601) position was reported to be associated with increased risk of autoimmune diseases including generalized vitiligo [5]. The aim of this study was to find out whether well-documented *PTPN22* 1858C/T polymorphism is associated with Gujarat vitiligo patients.

DNA was isolated from blood of 126 generalized vitiligo patients and 140 age-matched healthy controls. Oligonucleotide primers used for PCR amplification were 5′-GCCTCAATGAACTCCTCAA-3′ (forward primer) and 5′-CCTCCTGGGTTTGTACCTTA-3′ (reverse primer). Amplified PCR product (400 bp) was confirmed by agarose gel electrophoresis with 100 bp DNA ladder (MBI Fermentas).

For restriction fragment length polymorphism (RFLP) analysis, PCR-amplified products were digested with *Xcm*I (New England Biolabs). The amplicon with homozygous T/T allele of *PTPN22* is cleaved by *Xcm*I yielding 238 and162 bp fragments, whereas the amplicon with homozygous C/C allele remains uncut yielding 400 bp band, while the amplicon with heterozygous C/T allele yields three fragments of 400, 238 and 162 bp length (Fig. 1). The digested products were separated on 2% agarose gel along with 100 bp DNA ladder. Ethidium bromide stained gels were visualized under UV light.

The results of RFLP analysis of *PTPN22* 1858C/T polymorphism is shown in Table 1. The observed *PTPN22* allele frequencies of C and Twere 0.978 and 0.022, respectively, in controls; and 0.992 and 0.008, respectively, in vitiligo patients (Table 1). The allele frequencies of this C/T SNP did not differ significantly between the control and patient population ($p < 0.560$). When the observed control and patient genotype frequencies were compared with the expected values using a 3×2 contingency table in a standard χ^2-test (Table 1) they did not show any significant change ($p = 0.198$), suggesting that there is no association of the C/T (*Xcm*I) *PTPN22* marker with vitiligo. The observed genotype frequencies of the control population did not differ significantly from those predicted by Hardy Weinberg equation ($p < 0.932$). Also the patient population is not deviated from Hardy Weinberg equilibrium ($p < 0.994$).

PTPN22 gene is known to play an important role in negative T cell regulation. The 1858C/T polymorphism of *PTPN22* has been well documented for its association with several autoimmune diseases. However, our study shows that there is no association

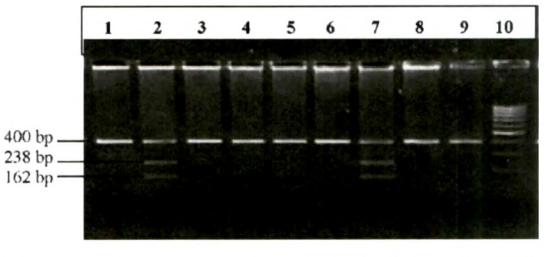

Fig. 1 PCR–RFLP analysis of *PTPN22* 1858C/T SNP: 2% agarose gel electrophoresis after overnight digestion of the PCR product with *Xcm*I. Lanes 1, 3, 4, 5, 6, 8, and 9 show subjects with homozygous alleles (C/C) with one intact band. Lanes 2 and 7 show subjects with heterozygous alleles (C/T) showing digestion of the 400 bp product into 238 bp and 162 bp bands. No subject with homozygous allele (T/T) was observed. Lane 10 shows a 100-bp ladder.

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Table 1 Distribution of genotypes and alleles for the 1858C/T SNP in *PTPN22* in vitiligo patients and control population.

	N	Observed genotype count			Observed allele frequencies		Expected genotype count			*p*-value
		C/C	C/T	T/T	C	T	C/C	C/T	T/T	
Controls	140	134	6	0	0.978	0.022	133.84	6.02	0.14	0.932
Patients	126	124	2	0	0.992	0.008	123.98	2.02	0.013	0.994
p-value						0.198				

between *PTPN22* 1858C/T polymorphism and vitiligo in Gujarat population. Canton et al. [5] have shown an association of *PTPN22* 1858C/T polymorphism with generalized vitiligo in Caucasian population. No other association studies are available on *PTPN22* 1858C/T polymorphism and vitiligo. There are several reports suggesting an association of 1858C/T polymorphism with other autoimmune diseases [6,7]. On the contrary, several reports also suggest a non-association of *PTPN22* 1858C/T polymorphism with autoimmune diseases [7–9]. These contradictory reports may be because of the differences in ethnicity of the studied populations. Ray et al. [8] have reported a non-association of *PTPN22* 1858C/T polymorphism with sporadic idiopathic hypoparathyroidism in Indian population.

PTPN22 gene encodes a lymphoid-specific phosphatase (Lyp). Lyp is an intracellular protein tyrosine phosphatase (PTP) and is physically bound to the SH3 domain of the Csk kinase through proline-rich motif and acts as an important suppressor of kinases that mediate T-cell activation. The ability of Csk and Lyp to inhibit T-cell receptor signaling requires their physical association. The C1858T substitution changes the amino acid from arginine (620R) to tryptophan (620W) at codon 620. This residue resides in the P1 proline-rich motif that is involved in binding to the SH3 domain of Csk. *In vitro* experiments have shown that the T-allele of *PTPN22* binds less efficiently to Csk than the C-allele does, suggesting that T-cells expressing the T-allele may be hyper-responsive, and consequently, individuals carrying this allele may be prone to autoimmunity [6,7].

The exact genetic mechanism of autoimmune diseases is not yet well understood. Autoimmune diseases are also influenced by environmental factors in addition to genetic factors. The study could not attain statistical significance consequent to lower prevalence rate of 1858T allele in the studied population. Interestingly, 1858T allele has been found to be absent in East Asians, including Japanese [9], Chinese [7], and South Asian Indians [8].

The present study shows that *PTPN22* 1858C/T polymorphism may not be associated with Gujarat vitiligo patients. Our studies on catalase exon 9 T/C and glutathione peroxidase codon 200 C/T polymorphisms also have not shown any significant association with vitiligo [10]. These results suggest for the presence of novel SNPs in Gujarat vitiligo population. This is the first report on *PTPN22* 1858C/T polymorphism in Gujarat vitiligo patients where the prevalence of vitiligo is alarmingly high.

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LETTER TO THE EDITOR

The *ACE* gene I/D polymorphism is not associated with generalized vitiligo susceptibility in Gujarat population

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Dear Sir,

Vitiligo is a hypomelanotic disease characterized by circumscribed depigmented macules and affects 0.5–2% of the world population (Passeron and Ortonne, 2005). However, in India Gujarat has the highest prevalence i.e., approximately 8.8% (Valia and Dutta, 1996). Vitiligo is hypothesized to be of autoimmune origin and genetic factors may also involve in precipitating this disease. Our earlier study suggests that 13.7% of Gujarat vitiliginous patients have at least one first-degree relative affected (Shajil et al., 2006). Angiotensin-converting enzyme (ACE), a key enzyme in the renin–angiotensin system, plays an important role in the physiology of the vasculature, blood pressure and inflammation. *ACE* has also been found to be associated with several autoimmune disorders (Scholzen et al., 2003; Vuk-Pavlovic and Rohrbach, 1988). The *ACE* gene is located on chromosome 17q23.3 and an insertion/deletion (I/D) polymorphism of a 287-base pair repetitive sequence in intron 16 of the *ACE* gene (NCBI: AF118569; repeat region 14094–14381) is reported to be associated with the development of vitiligo (Jin et al., 2004). The I/D polymorphism of the *ACE* gene accounts for the variability of serum ACE activity, D/D genotype having the highest and I/I genotype having the lowest ACE activity (Rigat et al., 1990). The aim of this study was to investigate the distribution of *ACE* I/D genotypes in generalized vitiligo patients and in healthy controls of Gujarat population to find the relationship between *ACE* I/D polymorphism and vitiligo.

A total of 125 generalized vitiligo patients and 156 ethnically matched healthy controls of Gujarat population were examined for *ACE* I/D polymorphism. The patients selected for this study had no other associated diseases. The DNA was prepared from blood samples after written informed consent was obtained. Oligonucleotide primers used for *ACE* intron 16 PCR amplification were 5′-CTGGAGACCACTCCCATCCTTTCT 3′-(forward primer) and 5′-GATGTGGCCATCACATTCGTCAGAT 3′ (reverse primer).

PCR amplification was performed in 25 μl reaction system containing 50 ng of genomic DNA and 20 pmol of each primer under the following conditions: 94°C for 10 min, 94°C for 30 s, 58°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. Amplified PCR products were analyzed on 2% agarose gel electrophoresis with 100 bp DNA ladder to identify the three genotype patterns: I/I (a 480 bp fragment), D/D (a 193 bp fragment) and I/D (two fragments, i.e., 480 and 193 bp). The distribution of the *ACE* I/D genotypes for patients and control subjects were compared by using the chi-square test with 2×2 and 3×2 contingency tables and P-values less than 0.05 were considered statistically significant. The statistical power of the current study was determined by using the G * Power software (Faul et al., 2007).

The observed allele and genotype frequencies for the *ACE* I/D polymorphism in controls and patient population are shown in Table 1. No significant difference in the genotype frequencies of I/I, I/D and D/D genotypes was observed between vitiligo patients and control subjects ($P = 0.459$) (Table 1), suggesting that there is no association of the *ACE* I/D polymorphism with vitiligo. The observed *ACE* allele frequencies for I and D alleles were 0.56 and 0.44 in controls; and 0.61 and 0.39 in vitiligo patients, respectively (Table 1). The allele frequencies of *ACE* I/D polymorphism did not differ significantly between the controls and patient population ($P = 0.252$) (Table 1). The observed genotype frequencies of the vitiligo patients did not show significant difference as predicted by the Hardy–Weinberg equation ($P < 0.964$). Also the control population did not deviate from Hardy–Weinberg equilibrium ($P < 0.905$). This study has 86% statistical power for the effect size 0.2 to detect

Table 1. Distribution of alleles and genotypes for the I/D polymorphism in *ACE* intron 16 in Gujarat vitiligo patients and control population

		Observed genotype counts (%)			Observed allele frequencies		Expected genotype counts[a]			
	N	I/I	I/D	D/D	I	D	I/I	I/D	D/D	P-value
Controls	156	51 (32.7)	74 (47.4)	31 (19.9)	0.56	0.44	49.6	76.8	29.6	0.905
Patients	125	46 (36.8)	61 (48.8)	18 (14.4)	0.61	0.39	46.9	59.4	18.9	0.964
P-value			0.459[b]			0.252[c]				
Odds ratio (95% CI)	0.821 (0.585–1.151)									

[a]Observed versus expected according to the Hardy–Weinberg equation.
[b]Controls versus patients using the chi-square test with 3 × 2 contingency table.
[c]Controls versus patients using the chi-square test with 2 × 2 contingency table.

association of *ACE* I/D polymorphism at $P < 0.05$ in patients and control population.

Autoimmunity plays a major role in the pathogenesis of vitiligo (Kemp et al., 2001). Autoimmune diseases are also influenced by environmental factors in addition to genetic factors (Zhang et al., 2004). ACE and its related substrates or products are known to have various functions in the immune system (Vuk-Pavlovic and Rohrbach, 1988). However, its underlying pathogenic mechanism in autoimmune diseases is yet to be understood. Previously, the D allele of the *ACE* gene was reported to confer susceptibility to vitiligo in Korean population (Jin et al., 2004). However, the current study suggests that the polymorphic variant D allele is not associated with vitiligo susceptibility. This may be because of the differences in ethnicity and/or geographic location of the subjects under study. Our results are comparable to the previous report in which genotype frequencies for the I/D polymorphism were not significantly different between controls and generalized vitiligo patient population (Akhtar et al., 2005). Our results on the distribution rate of I/D > I/I > D/D in the control population are in accordance with the previously reported distribution rate of I/D for Chinese (Lee, 1994), Japanese (Ishigami et al., 1995) and Korean population (Jin et al., 2004). This is the first report on the association of the *ACE* polymorphism with vitiligo in Gujarat population where prevalence of vitiligo is alarmingly high, and interestingly, the *ACE* gene I/D polymorphism may not play a role in the development of generalized vitiligo in Gujarat population.

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Lack of genetic association of promoter and structural variants of mannan-binding lectin (*MBL2*) gene with susceptibility to generalized vitiligo

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Summary

Background Vitiligo is a common depigmenting disorder resulting from the loss of functional melanocytes in the skin. It is hypothesized to be of autoimmune origin. Mannan-binding lectin (MBL) plays an important role in innate immunity. It helps in the clearance of apoptotic cells and in complement activation. Genetic variability due to structural and promoter polymorphisms in the MBL2 gene has been reported to be associated with increased risk for several autoimmune diseases including vitiligo.

Objectives The aim of this study was to explore whether MBL2 structural and promoter polymorphisms are associated with generalized vitiligo in Gujarat where the prevalence of vitiligo is alarmingly high.

Materials and methods We undertook a case–control study to investigate the association of MBL2 gene exon 1 polymorphisms – codon 52, codon 54 and codon 57 as well as promoter −221 polymorphism in 92 patients with generalized vitiligo and 94 unaffected age-matched controls by polymerase chain reaction-heteroduplex analysis.

Results The genotype and allele frequencies of MBL2 structural and promoter polymorphisms did not differ significantly between the control and patient population (P-values: $P < 0{\cdot}019$ for codon 52, $P < 0{\cdot}373$ for codon 54, $P < 0{\cdot}855$ for codon 57 and $P < 0{\cdot}889$ for −221 promoter polymorphisms) after Bonferroni's correction for multiple testing, which suggests that there is no association of MBL2 structural and promoter polymorphisms with generalized vitiligo.

Conclusions Our results suggest that the well-documented structural and promoter polymorphisms of the MBL2 gene may not be associated with generalized vitiligo in the Gujarat population.

Vitiligo is an acquired hypomelanotic disorder characterized by circumscribed depigmented macules in the skin resulting from the loss of functional melanocytes from the cutaneous epidermis. This is a cosmetic disfigurement disorder and may lead to psychological and social problems, particularly in brown and black people. It affects 0·5–1% of the world population.[1] In India the incidence of vitiligo is found to be 0·5–2·5%[2] whereas Gujarat and Rajasthan states have the highest prevalence, ~8·8%.[3] The aetiology of vitiligo is still unknown, but it is hypothesized to be of autoimmune origin due to its frequent association with several autoimmune diseases, the presence of circulating autoantibodies and autoreactive T cells in the sera of patients with vitiligo compared with unaffected individuals.[4,5]

Various factors such as oxidative stress, genetic factors, neural factors, toxic metabolites and lack of melanocyte growth factors might contribute to precipitating the disease in susceptible people.[6] The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association.[7] It has been found that about 20% of patients with vitiligo have at least one first-degree relative affected.[8] Our study also showed that 22% of Gujarat vitiliginous patients exhibit positive family history and 14% of patients have at least one first-degree relative affected.[4] The disease does not follow the simple Mendelian inheritance pattern and its mode of heredity suggests that it is a polygenic disease.

Furthermore, several genes that play a role in regulating immunity have been associated with susceptibility to vitiligo

including allelic variants in the cytotoxic T-lymphocyte antigen-4 gene (*CTLA4*),[9–11] the autoimmune susceptibility loci *AIS1*, *AIS2*, *AIS3* and *SLEV1*,[12–14] the autoimmune regulator (*AIRE*) gene,[15] NACHT leucine-rich repeat protein 1 (*NALP1* – maps at *SLEV1* susceptibility locus),[16,17] protein tyrosine phosphatase nonreceptor type 22 (PTPN22)[18–20] and certain human leucocyte antigen specificities of the major histocompatibility complex.[21,22]

Mannan-binding lectin (MBL) [synonyms: mannose-binding lectin (MBL), mannose-binding protein (MBP), mannan-binding protein (MBP)] is a liver-derived calcium-dependent serum protein, which plays an important role in innate immune defence. MBL is a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure resembling C1q of the complement pathway. MBL binds to cell-surface ligands such as mannose, *N*-acetylglucosamine residues, which are expressed on a wide range of microorganisms. MBL binds to carbohydrate moieties on microorganisms and thus leads to activation of the complement system via the lectin pathway.[23] In addition to complement activation, the protein has several distinct functions including promotion of complement-independent opsonophagocytosis, modulation of inflammation and promotion of apoptosis.[24] The role of the MBL pathway in complement activation and in the clearance of apoptotic cells suggests that genetic variability in MBL may be involved in the pathogenesis of autoimmune diseases.[25] A possible role of MBL in vitiligo pathogenesis could be due to defective clearance of apoptotic cells, which may contribute substantially to the triggering of autoimmune responses.[26] Also, MBL is involved in inflammatory response, regulation of proinflammatory cytokines, controlling adhesion molecules on inflammatory cells and immune complex removal. Thus, these studies emphasize the importance of MBL in cutaneous autoimmunity.[27]

The functional *MBL2* gene is located on chromosome 10 (q11.2–q21) and comprises four exons. Exon 1 encodes the signal peptide, a cysteine-rich region and part of the glycine-rich collagenous region. Three functional single-nucleotide polymorphisms (SNPs) in exon 1 of the *MBL2* gene have been reported: codon 54 (GGC > GAC; designated B allele), codon 57 (GGA > GAA; designated C allele) and codon 52 (CGT > TGT; designated D allele).[28–30] These variant alleles in the collagen-like domain cause structural changes in MBL and will lead to its low plasma levels and thus results in plasma concentrations varying by more than 1000-fold within the population (5 ng mL^{-1} to 5 μg mL^{-1}).[31,32] The promoter region of the *MBL2* gene contains a number of regulatory elements, which affect transcription of the gene and hence polymorphisms in the promoter region, thus affecting the plasma MBL concentration directly. Besides these exon 1 variant alleles, SNPs at promoters −550 (G/C; allele L) and −221 (G/C; allele X) have also been associated with low plasma levels of MBL.[33]

Recently, an association between *MBL2* gene codon 54 (allele B) polymorphism and susceptibility to vitiligo has been described in a Turkish population.[34] The aim of this study was to investigate the association of MBL2-deficient genotypes of both promoter and exon 1 with vitiligo susceptibility in the Gujarat population.

Materials and methods

Study population

A total of 92 patients with generalized vitiligo (mean age 28·37 ± 1·740; 29 males and 63 females) and 94 unaffected age-matched controls (mean age 27·28 ± 0·841; 60 males and 34 females) of the Gujarat population participated in this study. Sixty-two patients and 72 age-matched controls were genotyped for codon 52 (allele D), 84 patients and 81 controls were genotyped for codon 54 (allele B), and 68 patients and 72 controls were genotyped for codon 57 (allele C). For genotyping of the −221 promoter polymorphism, 92 patients and 93 control subjects were used. The study plan was approved by the ethical committee of the Department of Biochemistry, M.S. University of Baroda, Vadodara, Gujarat. The importance of the study was explained to all participants and written consent was obtained from the patients.

Methods

Genomic DNA was prepared from venous blood of patients with vitiligo and controls using a 'whole blood DNA extraction kit' (Bangalore Genei, Bangalore, India) according to the manufacturer's instructions. The exon 1 structural and promoter polymorphisms were genotyped by the polymerase chain reaction (PCR)-heteroduplex analysis technique. This technique involves the use of a universal heteroduplex generator (UHG), a synthetic DNA molecule based on the target genomic sequence.[35] The genomic DNA PCR product was combined with the UHG-PCR product and the two were allowed to anneal to produce characteristic heteroduplexes for different alleles. These heteroduplexes displayed different electrophoretic mobilities on a polyacrylamide gel enabling identification of a subject's genotype.[35]

Polymerase chain reaction amplification of genomic DNA and universal heteroduplex generator

Two separate PCRs were performed on genomic DNA samples to amplify exon 1 and −221 (X/Y) promoter regions by using forward (5′-CCAACACGTACCTGGTTCC-3′) and reverse (5′-CTGTGACCTGTGAGGATG-C-3′) primers for exon 1 and forward (5′-AGGCATAAGCCAGCTGGCAAT-3′) and reverse (5′-CTAAGGAGGGGTTCATCTG-3′) primers for −221 (X/Y) promoter region.

All primers were synthesized from MWG-Biotech, Germany. The PCR was performed in a 20-μL system containing 50 ng genomic DNA, 0·25 mmol L^{-1} dNTPs, 20 pmol of each primer, 2 mmol L^{-1} $MgCl_2$, PCR gold buffer and 1·5 U Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.). PCR amplification was carried out in a thermal cycler

(ABI 9700; Applied Biosystems) with the following conditions: initial denaturation at 95 °C for 3 min followed by denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 7 min. For the synthesis of each UHG, the same PCR conditions were used with each 50 ng UHG template (template size of 133 bp for exon 1 and 111 bp for −221 promoter region), which are commercially synthesized UHG (MWG-Biotech AG, Ebersberg, Germany), specifically designed to detect the exon 1 and promoter regions of MBL2. PCR products were analysed on a 2% agarose gel prior to heteroduplexing and confirmed with 100 bp DNA ladder (MBI Fermentas, Burlington, ON, Canada). Confirmation of specific amplification of genomic and UHG DNA using 2% agarose gel electrophoresis enabled comparison of the relative amounts of DNA obtained from each sample reaction against those from the UHG reaction.

Heteroduplex analysis

A mixture of approximately equal amounts of genomic and UHG-PCR products were combined prior to heating to 95 °C for 5 min and then allowed to cool at room temperature over 2 h to enable greater specific annealing. The PCR mix was spun briefly and the appropriate volume of 6 × loading dye was added to each mix. Heteroduplexes thus formed were checked by 20% polyacrylamide gel electrophoresis followed by ethidium bromide staining (Figs 1, 2).

On heteroduplexing, homozygotes developed two heteroduplex bands and heterozygotes generated four heteroduplex bands (Figs 1, 2). The exception was WT/R52C, where only three bands were generated due to co-migration of two heteroduplexes as a single upper band[35] (WT designating one wild-type allele and R52C, G54D and G57E, indicating an allele with a mutation at codons 52, 54 and 57, respectively) (Fig. 1). Interpretation of band patterns was done manually with the aid of known samples used as a reference grid. The genotype WT/G57E generating four bands spanning the whole gel was taken as the reference, and was included in each run. The position of all the other bands could be judged using this sample as the reference.

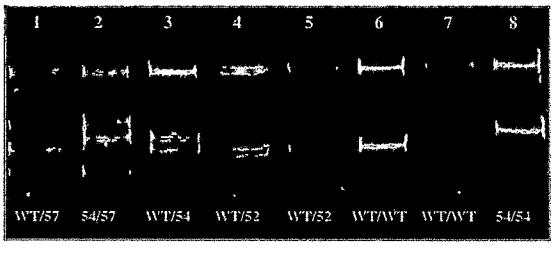

Fig 1. Polymerase chain reaction-heteroduplex analysis of MBL2 exon 1 (codon 52, codon 54 and codon 57) polymorphisms on 20% polyacrylamide gel electrophoresis: lane 1 shows heterozygous WT/57; lane 2 shows heterozygous 54/57; lane 3 shows heterozygous WT/54; lanes 4, 5 show heterozygous WT/52; lanes 6, 7 show homozygous WT/WT and lane 8 shows homozygous 54/54 individual.

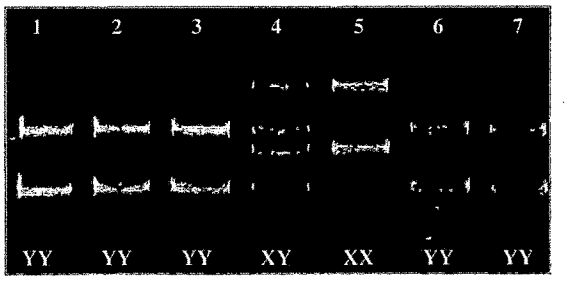

Fig 2. Polymerase chain reaction-heteroduplex analysis of MBL2 −221 (X/Y) promoter polymorphism on 20% polyacrylamide gel electrophoresis: lanes 1, 2, 3, 6 and 7 show homozygous YY; lane 4 shows heterozygous XY and lane 5 shows homozygous XX individual.

Statistical analysis

The distribution of the MBL2 genotypes and allele frequencies for patients and control subjects were compared using the χ^2 test with 3 × 2 and 2 × 2 contingency tables, respectively, using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, U.S.A.). The results were supported by odds ratios and 95% confidence intervals when appropriate. P-values < 0·0125 were considered as statistically significant due to Bonferroni's correction for multiple testing.

Results

We have investigated 92 patients with generalized vitiligo and 94 controls for exon 1 structural and promoter polymorphisms.

Genotyping of *MBL2* exon 1 structural polymorphisms

Codon 52, codon 54 and codon 57 polymorphisms of MBL2 exon 1 were not found to be associated in patients with generalized vitiligo ($P < 0{\cdot}019$ for codon 52, $P < 0{\cdot}373$ for codon 54, $P < 0{\cdot}855$ for codon 57) when genotypes were compared with the χ^2 test 3 × 2 contingency table (Table 1). Also, there was no significant difference in allele frequencies of codon 52, codon 54 and codon 57 between patients and controls when compared with the 2 × 2 contingency table ($P < 0{\cdot}020$ for D allele, $P < 0{\cdot}378$ for B allele and $P < 0{\cdot}858$ for C allele) (Table 1). Both the patient and control populations were found to be in Hardy–Weinberg equilibrium for all three exon 1 codon polymorphisms ($P < 1{\cdot}000$ and $P < 0{\cdot}932$, respectively, for codon 52 polymorphism; $P < 0{\cdot}869$ and $P < 0{\cdot}650$, respectively for codon 54 polymorphism; and $P < 0{\cdot}932$ and $P < 0{\cdot}909$, respectively, for codon 57 polymorphism) (Table 1). The genotypes for MBL2 structural polymorphisms were confirmed by running heteroduplexes on 20% polyacrylamide gel (Fig. 1).

The association study for exon 1 polymorphisms was also done by A/O genotyping where all three codon polymorphisms were placed in one group designated as 'O' allele and wild type as 'A' allele. The A/O genotypes were compared between controls and patients using the χ^2 3 × 2 contingency table.

Table 1 Association studies for mannan-binding lectin gene (MBL2) exon 1 polymorphisms in Gujarat patients with generalized vitiligo

SNP	Genotype or allele	Patients with vitiligo	Controls	P for association	P for HWE	Odds ratio (95% CI)
rs5030737, codon 52 (allele D)						
	Genotype	(n = 62)	(n = 72)			
	TT	0	0			
	CT	0	6	0·0192	1·0 (P); 0·932 (C)	0·08 (0·004–1·462)
	CC	62	65			
	Allele					
	T	0	8	0·007 (0·02[a])		
	C	124	136			
rs1800450, codon 54 (allele B)						
	Genotype	(n = 84)	(n = 81)			
	AA	1	0			
	AG	21	15	0·373	0·869 (P); 0·650 (C)	1·353 (0·694–2·639)
	GG	62	65			
	Allele					
	A	23	17	0·378		
	G	145	145			
rs1800451, codon 57 (allele C)						
	Genotype	(n = 68)	(n = 72)			
	AA	0	0			
	AG	6	7	0·855	0·932 (P); 0·909 (C)	0·899 (0·286–2·823)
	GG	62	65			
	Allele					
	A	6	7	0·858		
	G	130	137			
Genotype A/O[b]						
	Genotype	(n = 90)	(n = 94)			
	OO	1	1			
	AO	27	28	0·999	0·579 (P); 0·563 (C)	0·989 (0·566–1·726)
	AA	62	65			
	Allele					
	A	151	158	0·968		
	O	29	30			

[a]Yate's corrected P-value. SNP, single-nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium; [b]A, designation for wild-type alleles; O, common designation for the variant alleles B, C and D; (P), patients; (C), controls.

Interestingly, there was no significant association found between the A/O genotype and generalized vitiligo ($P < 0·999$) (Table 1). Furthermore, the 'O' allele was also not significantly associated with any of the population when compared using the 2×2 contingency table ($P < 0·968$) (Table 1).

Genotyping of *MBL2* –221 promoter polymorphism

In addition, we have also genotyped the –221 (X/Y) promoter polymorphism of the MBL2 gene in 92 patients and 93 controls. The genotypes for the –221 promoter polymorphism were confirmed by running heteroduplexes on 20% polyacrylamide gel (Fig. 2). There was no significant association found between the –221 promoter polymorphism and the risk of vitiligo when genotype frequencies of patients and controls were compared with the χ^2 test 3×2 contingency table ($P < 0·889$) (Table 2). Also, the allele frequencies of patients and controls for the –221 (X/Y) promoter polymorphism did not show significant difference when compared with the 2×2 contingency table ($P < 0·765$) (Table 2). However, both the control and patient populations were in accordance with Hardy–Weinberg equilibrium ($P < 0·120$ and $P < 0·370$, respectively) (Table 2).

Haplotype analysis

The promoter and structural genotypes of patients and controls were combined to construct haplotypes and to find out whether there was any haplotype associated with vitiligo susceptibility. Interestingly, no haplotype was found to be

Table 2 Distribution of alleles and genotypes for the −221 (X/Y; rs7096206) promoter polymorphism of MBL2 gene in patients with generalized vitiligo and controls

		Observed genotype counts (%)			Observed allele frequencies		Expected genotype counts[a]			
	n	X/X	X/Y	Y/Y	X	Y	X/X	X/Y	Y/Y	P-value
Controls	93	10 (10·75)	28 (30·10)	55 (59·13)	0·26	0·74	6·23	35·71	51·06	0·120
Patients	92	8 (8·70)	29 (31·52)	55 (59·78)	0·24	0·76	5·52	34·04	52·44	0·370
P-value			0·889[b]			0·765[c]				
OR (95% CI)			0·931 (0·582–1·489)							

[a]Observed vs. expected according to the Hardy–Weinberg equation; [b]controls vs. patients using the χ^2 test with 3 × 2 contingency table; [c]controls vs. patients using the χ^2 test with 2 × 2 contingency table. CI, confidence interval; OR, odds ratio.

Table 3 Distribution of mannan-binding lectin (MBL2) gene combined (structural + promoter) genotypes among patients with generalized vitiligo and controls

Combined genotype (structural + promoter)	Patients with vitiligo n = 90 (%)	Controls n = 88 (%)	P for association
YA/YA	33 (36·67)	34 (38·63)	
YA/XA	21 (23·34)	16 (18·18)	
YA/YO	20 (22·22)	19 (21·59)	
			0·962
XA/XA	8 (8·89)	9 (10·23)	
XA/YO	7 (7·78)	9 (10·23)	
YO/YO	1 (1·11)	1 (1·14)	
MBL high (YA/YA, YA/XA)	54 (60·00)	50 (56·82)	
MBL medium (XA/XA, YA/YO)	28 (31·11)	28 (31·82)	0·838
MBL low (XA/YO, YO/YO)	8 (8·89)	10 (11·36)	

MBL, mannan-binding lectin.

associated with generalized vitiligo when compared with the χ^2 test ($P < 0·962$) (Table 3).

Moreover, the haplotypes were distributed to represent MBL levels as high (YA/YA, YA/XA), medium (XA/XA, YA/YO) and low (XA/YO, YO/YO) in both controls and patients. However, no significant difference was found in the distribution of high, medium and low haplotypes when compared with the 2 × 2 contingency table ($P < 0·838$) (Table 3).

Discussion

Vitiligo susceptibility is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo as it shows frequent association with other autoimmune disorders such as rheumatoid arthritis, Graves' disease, etc.[36] One of the important regulators of innate immunity is MBL2, which is involved in complement activation and clearance of apoptotic cells suggestive of its importance in the pathogenesis of autoimmune diseases. We therefore selected MBL2 as a candidate gene to study vitiligo susceptibility in the Gujarat population and here we report that there is no association of MBL2 structural and promoter polymorphisms. Our previous studies on PTPN22 1858C/T (rs2476601) and angiotensin-converting enzyme (ACE) intron 16 I/D (NCBI: AF118569) polymorphisms did not show any significant association with vitiligo in the Gujarat population.[37,38]

MBL is a pattern-recognition molecule of the innate immune system and primarily recognizes specific sugar groups on the surface of microorganisms, enabling it to distinguish self from nonself. The three structural variants in exon 1 of the MBL2 gene are associated with significantly decreased levels of MBL compared with homozygotes of the wild-type gene.[39] The structural alterations in MBL due to three coding polymorphisms disrupt interactions of MBL with the associated serine proteases, thereby greatly diminishing complement-activating ability.[39,40]

The MBL concentration is also regulated by two additional promoter polymorphisms, namely −550 (G > C; allele L) and −221 (G > C; allele X), which directly affect the expression of the MBL gene. Because MBL is one of the proteins shown to be involved in the clearance of apoptotic cells, Werth *et al.*[26] suggested that any defect in the clearance of apoptotic cells may result in aggravation of autoimmune responses. In addition, MBL is also involved in inflammatory response, regulation of proinflammatory cytokines, controlling adhesion molecules on inflammatory cells and immune complex removal, thus emphasizing its importance in cutaneous autoimmunity.[27] Reports from Schallreuter *et al.*[41,42] showed a perturbed calcium homeostasis in vitiliginous melanocyte and keratinocyte cell cultures, suggesting that patients with vitiligo carrying functional and promoter polymorphisms may have defective calcium uptake resulting in low MBL concentration.

MBL2 gene polymorphisms have been associated with increased risk of autoimmune diseases such as systemic lupus erythematosus (SLE) in both Caucasoid and Chinese populations,[43,44] cutaneous lupus erythematosus and dermatomyositis.[26] Senaldi *et al.*[45] suggested that patients with SLE had a higher frequency of low levels of MBL than controls. Wang *et al.*[46] reported that codon 54 (allele B) of the MBL2 gene

might be associated with genetic susceptibility to Sjögren's syndrome (SJS). Thus most of the studies show that MBL deficiency is associated with poorer outcome as judged by clinical, inflammatory and radiographic indices.[47,48]

However, evaluation of MBL2 coding mutations in a Japanese population found no association with SLE and rheumatoid arthritis (RA).[49] Recent studies suggest that there is a greater prevalence of MBL deficiency in patients with RA[47] but other studies refute this assertion.[50] Rector *et al.*[51] found no association between structural polymorphisms in exon 1 of the MBL2 gene and susceptibility to Crohn disease.

Because vitiligo is hypothesized to be of autoimmune origin, it becomes relevant to screen patients with vitiligo for the presence of MBL2 structural and promoter variants which may predispose an individual for vitiligo. Previously, an association of codon 54 (allele B) with vitiligo was observed in a Turkish population where only two codons, 54 and 57, were genotyped with a smaller sample size.[34] Hence, it was pertinent to study all such important polymorphisms of MBL2 with an adequate sample size and we found there was no association between vitiligo and MBL2 structural and promoter polymorphisms. The positive association of codon 54 polymorphism and susceptibility to vitiligo described by Onay *et al.*[34] may be due to differences in the ethnicity of the studied populations.

This is the first report that shows nonassociation of structural and promoter polymorphisms of the MBL2 gene with generalized vitiligo. In conclusion, it can be considered that the structural and promoter polymorphisms in the MBL2 gene may not confer a role in generalized vitiligo susceptibility of the Gujarat population.

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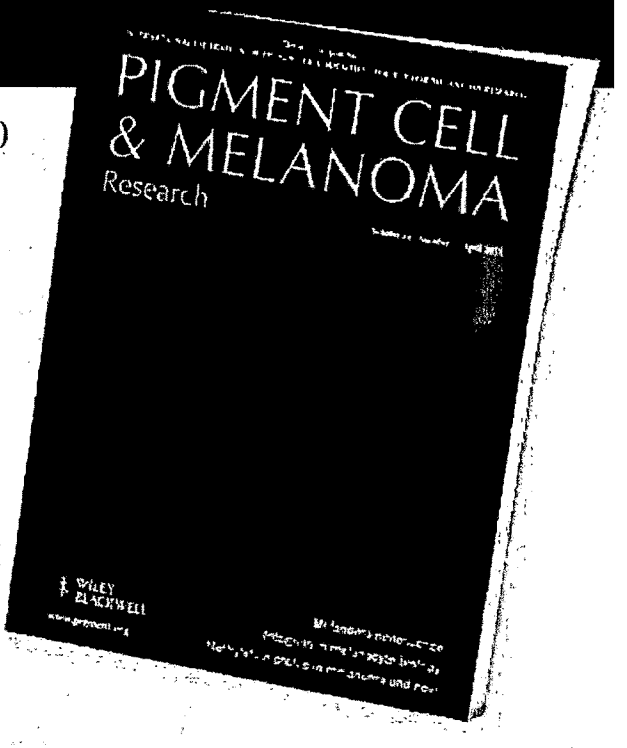

Cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) in isolated vitiligo: a genotype-phenotype correlation

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Cytotoxic T-lymphocyte-associated antigen-4 (*CTLA*-4) in isolated vitiligo: a genotype-phenotype correlation

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Dear Editor,

CTLA-4 is a negative regulator of T-cell function, which is suggested to be involved in susceptibility to autoimmune diseases including vitiligo, an acquired pigmentary disorder resulting from loss of melanocytes. Genetic variability in *CTLA*-4 gene is associated with its altered levels. *CTLA*-4 is expressed on activated $CD4^+$ and $CD8^+$ T cells and binds the same ligands, B7-1 and B7-2, as CD28 but with a 20–50-fold higher affinity. The CTLA-4:B7 interaction plays a critical role in regulating self-tolerance, and hence susceptibility to autoimmune diseases. The human *CTLA*-4 gene encodes two transcripts of 1.8 and 0.8 kb, known as full length *CTLA*-4 (fl*CTLA*-4) and soluble *CTLA*-4 (s*CTLA*-4) (Harper et al., 1991).

The aim of study was to explore exon 1 +49A/G (rs231775) and 3′ UTR CT60A/G (rs3087243) single nucleotide polymorphisms (SNPs) in *CTLA*-4 gene and to correlate them with *CTLA*-4 transcript levels in vitiligo patients and controls in Gujarat, India. The PCR-RFLP technique was used to genotype these polymorphisms (Supporting Information Figure S1) in 437 vitiligo patients and 746 controls. s*CTLA*-4 and fl*CTLA*-4 mRNA expression was assessed in 76 patients and 83 controls by real time PCR (Supporting Information Data S1, Tables S1 and S2).

CTLA-4 +49A/G polymorphism was not associated with vitiligo patients (P = 0.771); however, CT60A/G polymorphism showed a significant association (P = 0.0002) when genotypes were compared with a Chi-squared test 3 × 2 contingency table (Table 1). There was also no significant difference in the allele frequencies of +49A/G polymorphism (P = 0.461), whereas a significant difference in allele frequencies of CT60A/G polymorphism was observed (P = 0.0002) between patients and controls when compared with a 2 × 2 contingency table (Table 1). Both patient and control populations were in Hardy–Weinberg equilibrium for +49A/G polymorphism (P = 0.272 and P = 0.126, respectively). The control population was in Hardy–Weinberg equilibrium for CT60A/G polymorphism, whereas the patient population deviated from the equilibrium (P = 0.075 and P < 0.0001) (Table 1). This study has 95% statistical power for the effect size 0.1 to detect association of both the polymorphisms of *CTLA*-4 at P < 0.05 (Faul et al., 2007).

The two polymorphisms investigated were in moderate linkage disequilibrium (LD) (+49A/G: CT60A/G; $D' = 0.64$, $r^2 = 0.11$) as determined by HAPLOVIEW program version 4.1 (Barrett et al., 2005). The estimated frequencies of the haplotypes did not differ between patients and controls (global P-value = 0.123). However, the GG haplotype was more frequently observed in patients and increased the risk of vitiligo 1.2-fold [P = 0.048; odds ratio (OR): 1.243; 95% confidence interval (95%CI): (1.001–1.543)] (Supporting Information Table S3).

Expression of fl*CTLA*-4 and s*CTLA*-4 mRNA showed a significant decrease in patients after normalization with *GAPDH* expression (P = 0.007 and P = 0.037, respectively) (Figure 1A). The $2^{-\Delta\Delta Cp}$ analysis showed an approximately two-fold change in the expression of fl*CTLA*-4 and s*CTLA*-4 mRNA expression in patients as compared with controls (Figure 1B). The expression levels of fl*CTLA*-4 and s*CTLA*-4 for AA, AG and GG genotypes of +49A/G polymorphism did not differ significantly (Supporting Information Figure S2A,B). The expression levels of both fl*CTLA*-4 and s*CTLA*-4 differed significantly between patients and controls for CT60GG genotype (P = 0.004 and P = 0.005, respectively) but not for CT60AA genotype (P = 0.343 and P = 0.205, respectively) (Figure 1C,D).

The expression levels of fl*CTLA*-4 and s*CTLA*-4 were also analyzed with respect to haplotypes. Both fl*CTLA*-4 and s*CTLA*-4 mRNA levels were significantly decreased with AG haplotypes in patients as compared to controls (P = 0.036 and P = 0.020, respectively). The other three haplotypes, AA, GA and GG, did not differ with respect to fl*CTLA*-4 and s*CTLA*-4 expression levels (Figure 2A,B). We could not detect any significant difference in the s*CTLA*-4/fl*CTLA*-4 mRNA expression ratio between patients and controls (P = 0.346) (Figure S2C). However, +49AG genotype showed a decreased mRNA expression

Table 1. Association studies for *CTLA-4* gene exon 1 +49A/G and 3′ UTR CT60A/G polymorphisms in Gujarat vitiligo patients

SNP	Genotype or allele	Vitiligo patients (Freq.)	Controls (Freq.)	P for association	P for HWE	Odds ratio (95%CI)
	Genotype	(n = 347)	(n = 746)			
rs231775	AA	169 (0.49)	347 (0.47)	0.771	0.272 (Ps)	0.930 (0.766–1.128)
Exon 1	AG	140 (0.40)	310 (0.41)		0.126 (C)	
(+49A/G)	GG	38 (0.11)	89 (0.12)			
	Allele					
	A	478 (0.69)	1004 (0.67)	0.461		
	G	216 (0.31)	488 (0.33)			
	Genotype	(n = 437)	(n = 738)			
rs3087243	AA	146 (0.33)	256 (0.35)	0.0002*	<0.0001 (Ps)	1.417 (1.198–1.677)
3′ UTR	AG	135 (0.31)	337 (0.46)		0.075 (C)	
(CT60A/G)	GG	156 (0.36)	145 (0.19)			
	Allele					
	A	427 (0.49)	849 (0.58)	0.0002*		
	G	447 (0.51)	627 (0.42)			

n, number of patients/controls; HWE, Hardy–Weinberg equilibrium; Ps, patients; and C, controls. Values are significant at $P \leq 0.05$.
*Bonferroni's corrected P-value.

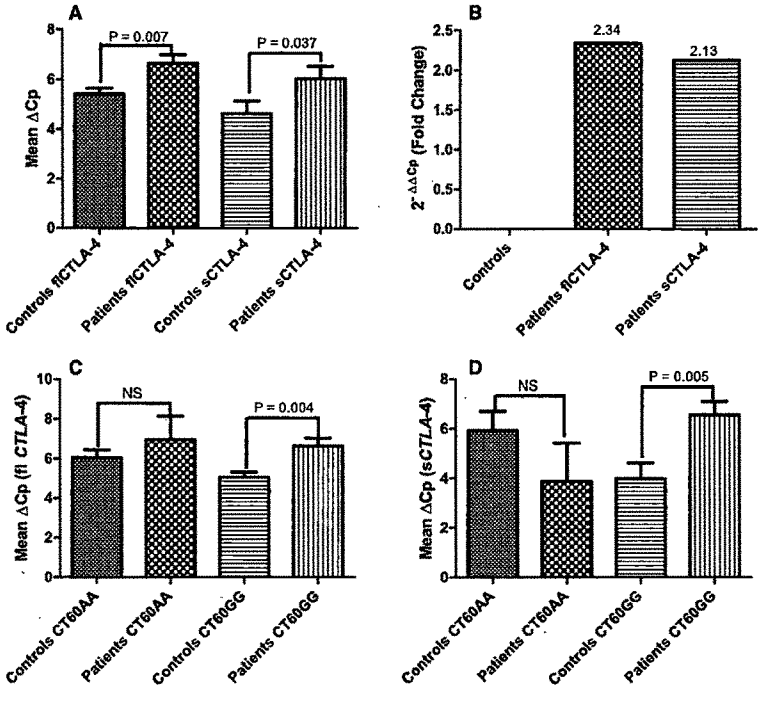

Figure 1. Relative gene expression of fl*CTLA-4* and s*CTLA-4* in 83 controls and 76 vitiligo patients. (A) Expression of fl*CTLA-4* and s*CTLA-4* mRNA as determined by mean ΔCp. Vitiligo patients showed significantly reduced mRNA levels of fl*CTLA-4* (P = 0.007) and s*CTLA-4* (P = 0.037) as compared to controls. (B) Expression fold change of fl*CTLA-4* and s*CTLA-4* showed approximately two-fold change as determined by the $2^{-\Delta\Delta Cp}$ method. (C) Relative mRNA expression of fl*CTLA-4* with respect to CT60A/G genotype. GG genotype showed a significant decrease in the levels of fl*CTLA-4* mRNA (P = 0.004) in patients as compared with AA genotype (P = 0.343) as determined by mean ΔCp. (D) Relative mRNA expression of s*CTLA-4* with respect to CT60A/G genotypes. GG genotype showed a significant decrease in levels of s*CTLA-4* mRNA (P = 0.005) in patients as compared with AA genotype (P = 0.205) as determined by mean ΔCp. NS, non-significant.

ratio in patients (P = 0.049), whereas the other two genotypes, +49AA and +49GG, did not differ (Figure 2C). When mRNA expression ratios for CT60A/G genotypes were compared, GG genotype showed a significant decrease in the ratio (P = 0.019) in patients; however, AA genotype did not differ significantly (Figure 2D). Moreover, the ratio of s*CTLA-4* and fl*CTLA-4* mRNA expression did not differ significantly for any of the haplotypes (AA, AG, GA, GG) (Figure S2D).

A few studies have addressed the possible functional significance of *CTLA-4* polymorphisms. Interestingly, the G allele of *CTLA-4* +49A/G SNP was reported to be involved in the altered intracellular transport of CTLA-4 protein and its availability on the cell surface. In addition, the 3′ UTR CT60A/G allelic variation was reported to be correlated with lower mRNA levels of the soluble alternative splice form of *CTLA-4* (s*CTLA-4*) (Ueda et al., 2003), which is in concordance with our results.

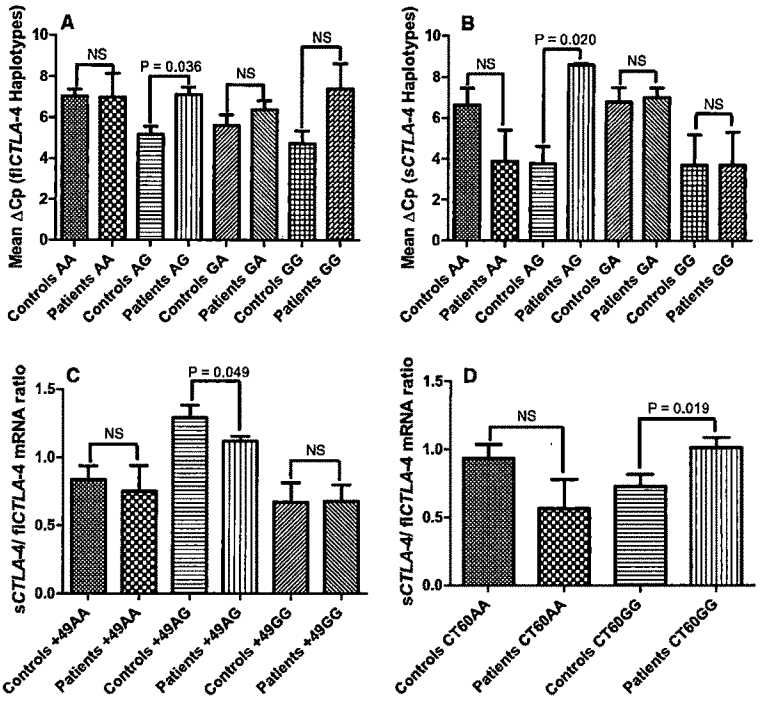

Figure 2. Relative mRNA expression of fl*CTLA*-4 and s*CTLA*-4 with respect to haplotypes and s*CTLA*-4/fl*CTLA*-4 mRNA ratios in 83 controls and 76 vitiligo patients. (A) AG haplotype showed a significant decrease in the levels of fl*CTLA*-4 mRNA in patients as compared with controls as determined by mean ΔCp (P = 0.036). The other three haplotypes, AA, GA and GG, did not differ in fl*CTLA*-4 expression in patients and controls (P = 0.955, P = 0.476 and P = 0.075, respectively). (B) AG haplotype showed a significant decrease in the levels of s*CTLA*-4 mRNA in patients as compared with controls as determined by mean ΔCp (P = 0.020). The other three haplotypes, AA, GA and GG, did not differ in fl*CTLA*-4 expression in patients and controls (P = 0.152, P = 0.865 and P = 0.992, respectively). (C) The s*CTLA*-4/fl*CTLA*-4 mRNA ratio was analyzed with respect to +49A/G genotypes in patients and controls. AG genotype showed a significant decrease in the ratio (P = 0.049) as compared to AA (P = 0.668) and GG (P = 0.964) genotypes. (D) The s*CTLA*-4/fl*CTLA*-4 mRNA ratio was analyzed with respect to CT60A/G genotypes in patients and controls. GG genotype showed a significant increase in the ratio (P = 0.019) as compared with AA (P = 0.096) genotype. NS, non-significant.

In the present study, we report an association between CT60A/G polymorphism and susceptibility to vitiligo. However, studies from Caucasian and Romanian populations did not find this association with vitiligo (Birlea et al., 2009; LaBerge et al., 2008). Another study from a Caucasian population only showed a significant association of the polymorphism with vitiligo patients who had other autoimmune diseases (Blomhoff et al., 2005). We did not find an association of genotype and allele frequencies for +49A/G polymorphism with vitiligo patients and our results are in line with those of Romanian and South Indian populations (Birlea et al., 2009; Deeba et al., 2010).

This is the first report where *CTLA*-4 expression levels were determined in vitiligo patients. Interestingly, we found decreased mRNA expression of both fl*CTLA*-4 and s*CTLA*-4 in patients and CT60G allele greatly reduced the mRNA expression of both isoforms, suggesting its crucial role in the pathogenesis of vitiligo. CT60AA genotype did not modulate *CTLA*-4 mRNA expression, suggesting that there may be other genetic factors involved in disease pathogenesis in patients harboring this genotype, evidence that vitiligo may have varied types of precipitating factors.

Further, decreased s*CTLA*-4 mRNA levels in patients with haplotype AG (+49A:CT60G) indicates the positive correlation of CT60G in vitiligo pathogenesis. Recently, Gerold et al. (2011) showed decreased s*CTLA*-4 levels in Type 1 diabetes, suggesting that lower s*CTLA*-4 expression may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. Moreover, an increased s*CTLA*-4/fl*CTLA*-4 mRNA ratio with +49AG and CT60GG genotypes in vitiligo patients suggests that G allele confers lower s*CTLA*-4 mRNA levels. These results are in agreement with those of Atabani et al. (2005). In contrast, Anjos et al. (2005) and Mayans et al. (2007) could not find an effect of the CT60 polymorphism on the expression of s*CTLA*-4 and fl*CTLA*-4.

In conclusion, our findings suggest that the dysregulated *CTLA*-4 expression in vitiligo patients could result, at least in part, from variations at the genetic level. For the first time, we show that the 3′ UTR CT60A/G poly-

morphism of the *CTLA*-4 gene influences both full length and soluble *CTLA*-4 mRNA levels in vitiligo patients. This genotype–phenotype correlation of *CTLA*-4 supports the autoimmune pathogenesis of vitiligo.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PCR-RFLP analysis of *CTLA*-4 exon 1 +49 A/G and 3′ UTR CT60A/G polymorphisms.

Figure S2. Relative mRNA expression of fl*CTLA*-4, s*CTLA*-4 and its ratios (s*CTLA*-4/fl*CTLA*-4) in 83 controls and 76 vitiligo patients.

Table S1. Demographic characteristics of vitiligo patients and unaffected controls.

Table S2. Primers used for *CTLA*-4 SNPs genotyping and gene expression analysis.

Table S3. Distribution of haplotypes frequencies for *CTLA*-4 gene polymorphisms (+49A/G and CT60A/G) among vitiligo patients and controls.

Data S1. Methods.

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ORIGINAL ARTICLE

See related commentary on pg 13

HLA Alleles and Amino-Acid Signatures of the Peptide-Binding Pockets of HLA Molecules in Vitiligo

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Vitiligo is a depigmenting disorder of the skin that is characterized by the loss of functional melanocytes from the lesional sites. Although the exact etiology is not understood, autoimmunity is thought to be a crucial deterministic factor. A recurring theme of several autoimmune disorders is the aberrant presentation of self-antigens to the immune system, which triggers downstream perturbations. Here we examine the role of alleles of HLA class I and class II loci to delineate vitiligo manifestation in two distinct populations. Our studies have identified three specific alleles, *HLA-A*33:01*, *HLA-B*44:03*, and *HLA-DRB1*07:01*, to be significantly increased in vitiligo patients as compared with controls in both the initial study on North Indians ($N=1,404$) and the replication study in Gujarat ($N=355$) cases, establishing their positive association with vitiligo. Both generalized and localized vitiligo have the same predisposing major histocompatibility complex alleles, i.e., *B*44:03* and *DRB1*07:01*, in both the populations studied, beside the differences in the frequencies of other alleles, suggesting that localized vitiligo too may be an autoimmune disorder. Significant differences in the amino-acid signatures of the peptide-binding pockets of HLA-A and HLA-B α-chain and HLA-DR β-chain were observed between vitiligo patients and unaffected controls.

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INTRODUCTION

Vitiligo is a multifactorial, depigmenting disorder of the skin characterized by loss of functional melanocytes, resulting in appearance of milky white patches on the skin. It is the most common pigmentation disorder affecting 0.5–1% of the world population (Taieb and Picardo, 2007). In India, the incidence varies from 0.25 to 2.5% (Das *et al.*, 1985; Handa and Kaur, 1999) in most ethnic groups, with 8.8% in populations from western states of Gujarat and Rajasthan (Shajil *et al.*, 2006). Vitiligo manifests in broadly two clinical subtypes: generalized and localized (Taieb and Picardo, 2007). Generalized vitiligo includes vitiligo vulgaris, acrofacial vitiligo, and vitiligo universalis. Localized vitiligo includes segmental and focal types (Taieb and Picardo, 2007). Precise etiology of vitiligo is not known; however, several hypotheses have been proposed, which include autoimmune (Kemp *et al.*, 2001), neural (Dell'anna and Picardo, 2006), autocytotoxic (Lerner, 1971), and genetic hypotheses (Bhatia *et al.*, 1992). Of these, autoimmune hypothesis remains most widely accepted because of frequent occurrence of other autoimmune diseases in vitiligo cases, the presence of autoreactive T cells in the vitiligenous lesions and peripheral circulation, and the presence of circulating autoantibodies in the sera of the patients (Schwartz and Janniger, 1997; Kovacs, 1998; Kemp *et al.*, 2001; Lang *et al.*, 2001; Wankowicz-Kalinska *et al.*, 2003; van den Boorn *et al.*, 2009; Waterman *et al.*, 2010). The frequency of vitiligo cases in first-degree relatives is ~7%, suggesting an important role of genetic factors in disease pathogenesis (Majumder *et al.*, 1993; Alkhateeb *et al.*, 2003).

The major histocompatibility complex (MHC) has been implicated in recent genome-wide association studies on vitiligo (Jin *et al.*, 2010; Quan *et al.*, 2010), in which several single-nucleotide polymorphisms in the MHC region were significantly associated with the disease. However, the authors imputed the HLA (MHC of man) *class I* alleles, based on the linkage disequilibrium (LD) of *HLA* alleles with the specific single-nucleotide polymorphisms, but could not impute *HLA class II* alleles because of limitations of *HLA* allele imputation in the Chinese Han from Beijing (China) samples (Quan *et al.*, 2010). MHC spans about 3.6 megabases on chromosome 6p21.3 of man (Horton *et al.*, 2004), with 224 known gene loci. *HLA* is the most

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[6]*Composition first described in* Hum Genet *2005, 118, 1–11*

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Abbreviations: CI, confidence interval; MHC, major histocompatibility complex; OR, odds ratio

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polymorphic system of the human genome, with 1,519 alleles for *HLA-A* locus, 2,069 alleles of *HLA-B* locus, 1,016 alleles for HLA-C locus, and 966 alleles for *HLA-DRB1*. With such a great diversity at each locus, LD of *HLA* alleles with diallelic single-nucleotide polymorphisms may be misleading, as several *HLA* alleles may be in LD with one or the alternate single-nucleotide polymorphism. Thus, the derivation based on these LDs may not be accurate, especially in the populations from which no previous *HLA* data are available. Thus, it is important to study the frequencies of HLA alleles in patients rather than imputing based on LD.

Many autoimmune diseases such as Type 1 diabetes (Todd *et al.*, 1987; Rani *et al.*, 2004) and rheumatoid arthritis (Winchester, 1994) have been shown to be associated with specific alleles of one or more genes of the *MHC*. The association of multiple *HLA* class I and class II antigens have been suggested for vitiligo (de Vijlder *et al.*, 2004; Zhang *et al.*, 2004a, b), although no consensus could be reached, because of distinct ethnic groups (Zamani *et al.*, 2001; Tastan *et al.*, 2004; Orozco-Topete *et al.*, 2005), small sample sizes, and low-resolution typing methods used to identify the HLA antigens. We report here a very robust study on a sample size of 1,404 vitiligo cases and 902 unaffected controls from North India for the alleles at *HLA-A*, *-B*, and *-C* (class I), and *-DRB1* (class II) loci using molecular methods. The replication study was conducted on 355 cases and 441 controls from Gujarat (Table 1). We also report differences in the amino-acid signatures of peptide-binding pockets of the HLA molecules in vitiligo patients as compared with controls.

RESULTS

HLA class I and *II* alleles and haplotypes in vitiligo cases as compared with unaffected controls

Diversity in the number and frequencies of HLA alleles was observed both in patients and controls. We observed 78, 160, 35, and 106 alleles for *HLA-A*, *-B*, *-C*, and *-DRB1* loci, respectively, in cases, and 68, 111, 44, and 94 alleles for *HLA-A*, *-B*, *-C*, and *-DRB1* loci, respectively, in unaffected controls from North India, and similar diversity was observed in the replication study. However, *HLA-A*33:01*, *B*44:03*, *C*07:01*, *DRB1*07:01*, and *DRB1*12:02* were significantly increased, and *HLA-A*26:01*, *A*31:01*, *B*08:01*, *B*51:01*, *C*12:03*, *DRB1*03:01*, *DRB1*11:01*, and *DRB1*15:02* were significantly reduced (Table 2 and Supplementary Table S1 online) in the vitiligo subjects from North India as compared with controls. In the replication study, we observed *HLA-A*33:01*, *B*44:03*, and *DRB1*07:01* to be significantly increased, and *DRB1*03:01* and *DRB1*11:01* to be significantly reduced in the cases as compared with ethnically matched controls (Table 2). These associations were significant, irrespective of the age at onset or the gender of the cases (Supplementary Tables S2–S5 online).

To determine which *HLA* alleles were significantly associated with vitiligo in the absence of *DRB1*07:01*, we deleted all the samples with *DRB1*07:01* from both the case and control groups and then analyzed the data. *B*37:01* and *DRB1*10:01* were significantly increased, whereas *B*08:01* and *DRB1*03:01* (Table 3) were significantly reduced in vitiligo cases in both initial and replication studies. In *A*33:01*-negative cases, *B*37:01*, *B*44:03*, *DRB1*07:01*, and *DRB1*10:01* were significantly increased and *DRB1*03:01* was significantly reduced as compared with *A*33:01*-negative controls in both initial and replication studies (Supplementary Table S6 online). In *B*44:03*-negative cases too, we observed a significant increase of *B*37:01*, *DRB1*07:01*, and *DRB1*10:01* and a significant decrease of *DRB1*03:01* as compared with *B*44:03*-negative controls in both the populations studied (Supplementary Table S7 online). *DRB1*07:01* remained significantly increased in *B*37:01*-negative patient samples also from North India ($P<5.69\times10^{-33}$, odds ratio (OR) = 3.03, 95% confidence interval (CI) = 2.51–3.66) and Gujarat ($P<1.33\times10^{-17}$, OR = 3.87, 95% CI = 2.78–5.39, data not shown).

HLA-C alleles were studied only in North Indian vitiligo cases ($N=404$) and healthy controls ($N=438$). We did not see any significant increase in *HLA-C* group 1 and group 2 alleles (based on Ser^{77}/Asn^{80} and Asn^{77}/Lys^{80}, respectively) in vitiligo as compared with controls (Supplementary Table S1 online). In the absence of *C*07:01*, which was significantly increased in vitiligo cases, *DRB1*07:01* still remained significantly increased (30% in vitiligo vs. 23.08% in controls) in cases as compared with controls ($P<0.047$, OR = 1.42, 95% CI = 0.99–2.06). However, in *DRB1*07:01*-negative samples, *C*07:01* does not remain significant (14.09% in vitiligo vs. 8.81% in controls) in vitiligo cases when compared with controls ($P<0.053$, OR = 1.69, 95% CI = 0.95–3.03), suggesting that association of *C*07:01* is probably because of it being in LD with *DRB1*07:01*.

Estimation of *HLA* haplotype frequencies was done using the expectation maximization algorithm (Excoffier and Slatkin, 1995; Excoffier *et al.*, 2005) with Arlequin Ver 3.5

Table 1. Description of vitiligo patients and controls used in the study

	Unaffected controls		Vitiligo patients	
Population	Unaffected controls (males, females, age mean ± SD)	Total vitiligo patients (males, females, age mean ± SD, age at onset mean ± SD)	Generalized vitiligo (males, females, age mean ± SD, age at onset mean ± SD)	Localized vitiligo (males, females, age mean ± SD, age at onset mean ± SD)
North India	902 (538, 364, 36.35 ± 14.75)	1,404 (838, 566, 28.72 ± 14.59, 22.15 ± 14.32)	1,097 (649, 448, 29.44 ± 15.04, 22.34 ± 14.61)	307 (189, 118, 26.16 ± 12.50, 21.46 ± 13.22)
Gujarat (replication study)	441 (264, 177, 28.04 ± 14.97)	355 (148, 207, 31.18 ± 15.03, 15.70 ± 12.65)	250 (107, 143, 32.06 ± 14.82, 16.14 ± 12.61)	105 (41, 64, 29.12 ± 15.37, 14.66 ± 12.76)

Table 2. *HLA-A, HLA-B,* and *HLA-DRB1* alleles showing significant differences in vitiligo patients as compared with unaffected controls from North India and Gujarat

	Vitiligo	Generalized	Localized	Controls	Vitiligo versus unaffected controls		Generalized vitiligo versus controls		Localized vitiligo versus controls	
HLA allele	No. (%)	No. (%)	No. (%)	No. (%)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
A*02:01 N India	197 (14.03)	160 (14.59)	37 (12.05)	88 (9.76)	0.002	1.50 (1.14–1.98)	0.001	1.57 (1.18–2.10)	0.25	1.26 (0.82–1.93)
Gujarat	27 (7.62)	17 (6.80)	10 (9.62)	44 (10.78)	0.13	0.68 (0.40–1.15)	0.087	0.60 (0.32–1.11)	0.72	0.88 (0.38–1.86)
*A*26:01* N India	83 (5.91)	64 (5.83)	19 (6.19)	90 (9.98)	0.00029	0.56 (0.41–0.78)	0.0005	0.56 (0.39–0.79)	0.045	0.59 (0.34–1.00)
Gujarat	21 (5.93)	15 (6.0)	6 (5.76)	36 (8.82)	0.13	0.65 (0.35–1.17)	0.18	0.66 (0.33–1.27)	0.31	0.63 (0.21–1.57)
*A*31:01* N India	43 (3.06)	32 (2.92)	11 (3.58)	58 (6.43)	0.0001	0.45 (0.30–0.70)	0.0002	0.43 (0.27–0.69)	0.063	0.54 (0.25–1.05)
Gujarat	16 (4.51)	13 (5.2)	3 (2.88)	18 (4.41)	0.94	1.02 (0.48–2.16)	0.64	1.18 (0.53–2.61)	0.35[1]	0.72 (0.31–1.65)[2]
*A*33:01* N India	353 (25.14)	276 (25.16)	77 (25.08)	104 (11.53)	1.21×10^{-15}	2.57 (2.02–3.29)	1.09×10^{-14}	2.57 (2.00–3.33)	9.01×10^{-9}	2.56 (1.82–3.61)
Gujarat	69 (19.49)	53 (21.2)	16 (15.38)	51 (12.5)	0.008	1.69 (1.12–2.56)	0.003	1.88 (1.20–2.93)	0.43	1.27 (0.65–2.40)
*A*68:01* N India	189 (13.46)	161 (14.68)	28 (9.12)	139 (15.41)	0.19	0.85 (0.66–1.09)	0.64	0.94 (0.73–1.21)	0.0058	0.55 (0.34–0.85)
Gujarat	71 (20.05)	55 (22.0)	16 (15.38)	46 (11.27)	0.0008	1.97 (1.29–3.02)	0.0002	2.21 (1.41–3.49)	0.25	1.43 (0.72–2.71)
*B*08:01* N India	68 (4.84)	53 (4.83)	15 (4.89)	112 (12.42)	3.68×10^{-11}	0.35 (0.25–0.49)	8.63×10^{-10}	0.35 (0.25–0.50)	0.0002	0.36 (0.19–0.64)
Gujarat	27 (7.64)	17 (6.85)	10 (9.52)	38 (9.33)	0.40	0.80 (0.46–1.38)	0.26	0.71 (0.37–1.33)	0.95	1.02 (0.43–2.18)
*B*37:01* N India	95 (6.77)	76 (6.93)	19 (6.19)	41 (4.55)	0.027	1.54 (1.03–2.27)	0.023	1.56 (1.04–2.37)	0.25	1.38 (0.76–2.49)
Gujarat	43 (12.18)	29 (11.69)	14 (13.33)	26 (6.38)	0.0056	2.03 (1.18–3.52)	0.018	1.94 (1.07–3.52)	0.018	2.25 (1.04–4.68)
*B*44:03* N India	419 (29.84)	347 (31.63)	72 (23.45)	97 (10.75)	7.05×10^{-27}	3.53 (2.76–4.53)	5.41×10^{-29}	3.83 (2.98–4.96)	2.98×10^{-8}	2.54 (1.78–3.61)
Gujarat	88 (24.92)	66 (26.61)	22 (20.95)	39 (9.58)	1.54×10^{-8}	3.13 (2.04–4.84)	8.29×10^{-9}	3.42 (2.17–5.42)	0.001	2.50 (1.33–4.58)
*B*51:01* N India	133 (9.47)	114 (10.39)	19 (6.19)	167 (18.51)	3.00×10^{-10}	0.46 (0.35–0.59)	2.00×10^{-7}	0.51 (0.39–0.66)	2.34×10^{-7}	0.29 (0.17–0.48)
Gujarat	46 (13.03)	33 (13.30)	13 (12.38)	45 (11.05)	0.40	1.20 (0.76–1.91)	0.38	1.23 (0.74–2.04)	0.70	1.13 (0.54–2.25)
*DRB1*03:01* N India	92 (6.55)	67 (6.11)	25 (8.14)	174 (19.29)	9.23×10^{-21}	0.29 (0.22–0.38)	2.10×10^{-19}	0.27 (0.19–0.37)	5.37×10^{-6}	0.37 (0.23–0.58)
Gujarat	22 (6.19)	14 (5.6)	8 (7.61)	61 (13.83)	0.00045	0.41 (0.23–0.70)	0.0008	0.37 (0.18–0.68)	0.08	0.51 (0.20–1.12)
*DRB1*07:01* N India	724 (51.57)	601 (54.79)	123 (40.07)	248 (27.49)	3.16×10^{-30}	2.80 (2.33–3.37)	1.10×10^{-34}	3.19 (2.63–3.87)	0.000037	1.76 (1.33–2.33)
Gujarat	184 (51.83)	133 (53.2)	51 (48.57)	111 (25.17)	9.81×10^{-15}	3.19 (2.34–4.36)	1.28×10^{-13}	3.37 (2.39–4.75)	2.38×10^{-6}	2.80 (1.76–4.45)
*DRB1*11:01* N India	87 (6.20)	64 (5.83)	23 (7.49)	93 (10.31)	0.0003	0.57 (0.41–0.78)	0.0002	0.53 (0.38–0.76)	0.14	0.70 (0.42–1.15)
Gujarat	19 (5.35)	16 (6.4)	3 (2.85)	57 (12.92)	0.0003	0.38 (0.21–0.67)	0.0073	0.46 (0.24–0.84)	0.0010[1]	0.22 (0.10–0.49)[2]
*DRB1*12:02* N India	67 (4.77)	53 (4.83)	14 (4.56)	14 (1.55)	0.00004	3.17 (1.75–6.16)	0.00005	3.22 (1.74–6.32)	0.0025	3.03 (1.32–6.93)
Gujarat	14 (3.94)	11 (4.4)	4 (3.80)	23 (5.21)	0.51	0.80 (0.38–1.63)	0.63	0.83 (0.36–1.82)	0.38[1]	0.78 (0.38–1.63)[2]
*DRB1*15:02* N India	165 (11.75)	117 (10.67)	48 (15.64)	152 (16.85)	0.0005	0.65 (0.51–0.84)	0.00005	0.59 (0.45–0.77)	0.62	0.91 (0.63–1.31)
Gujarat	54 (15.21)	37 (14.8)	17 (16.19)	82 (18.59)	0.20	0.78 (0.52–1.16)	0.20	0.76 (0.48–1.18)	0.56	0.84 (0.44–1.52)

Abbreviations: CI, confidence interval; N, North; OR, odds ratio.
[1]*P*-value calculated using Fisher's exact test.
[2]OR was calculated by using Woolf's method with Haldane's modification.
Vitiligo patient samples from North India (*N*=1,404; generalized (*n*=1,097), localized (*n*=307)) were compared with unaffected controls (*N*=902, North Indian origin) for *HLA-A, HLA-B,* and *HLA-DRB1* loci. Overall, 354 vitiligo patients, 250 generalized and 104 localized, were compared with 408 Gujarati controls for *HLA-A* locus. Overall, 353 vitiligo patients, 248 generalized and 105 localized, were compared with 407 Gujarati controls for *HLA-B* locus, and 355 vitiligo patients, 250 generalized and 105 localized, were compared with 441 Gujarati controls for *HLA-DRB1* locus.

(http://cmpg.unibe.ch/software/arlequin35/). Haplotype analysis for three loci showed that haplotypes *A*33:01-B*44:03-DRB1*07:01* and *A*24:02-B*44:03-DRB1*07:01* were significantly increased, and *A*26:01-B*080:01-DRB1*03:01* was significantly reduced in vitiligo patients in both initial and the replication studies (Table 4). Analysis of two-locus haplotypes revealed that (Table 5) haplotypes *A*33:01-DRB1*07:01* and *B*44:03-DRB1*07:01* were significantly increased, and *A*26:01-DRB1*03:01* was significantly reduced in vitiligo cases, in both initial and replication studies.

Four-locus haplotypes were constructed for North Indian samples and a significant increase of *A*33:01-C*07:01-B*44:03-DRB1*07:01* and *A*02:01-C*07:01-B*44:03-DRB1*07:01*, and a significant reduction in haplotype *A*26:01-C*07:02-B*08:01-DRB1*03:01* in vitiligo patients was observed as compared with controls (Supplementary Table S8 online).

HLA class I and II alleles and haplotypes in different manifestations of vitiligo: generalized and localized vitiligo as compared with unaffected controls

To study the association of *MHC* alleles with different manifestations of vitiligo, cases were divided into two clinical types—generalized and localized. *A*33:01, B*44:03, C*07:01,* and *DRB1*07:01* were significantly increased in

Table 3. *HLA-A*, *HLA-B*, and *HLA-DRB1* alleles showing significant differences in all vitiligo and generalized and localized vitiligo as compared with healthy controls in *DRB1*07:01*-negative samples in North Indian and Gujarat populations

	Vitiligo	Generalized vitiligo	Localized vitiligo	Controls	Vitiligo versus controls		Generalized vitiligo versus controls		Localized vitiligo versus controls	
HLA allele	No. (%)	No. (%)	No. (%)	No. (%)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
HLA-A										
*A*02:01* N India	92 (13.53)	70 (14.11)	22 (11.96)	68 (10.40)	0.078	1.34 (0.95–1.91)	0.055	1.41 (0.97–2.05)	0.54	1.17 (0.66–1.98)
Gujarat	13 (7.65)	8 (6.84)	5 (9.43)	42 (13.38)	0.058	0.53 (0.25–1.05)	0.059	0.47 (0.18–1.06)	0.29[1]	0.72 (0.37–1.41)[2]
*A*24:02* N India	146 (21.47)	105 (21.17)	41 (22.28)	143 (21.87)	0.86	0.97 (0.74–1.27)	0.77	0.95 (0.71–1.28)	0.90	1.02 (0.67–1.53)
Gujarat	32 (18.82)	23 (19.66)	9 (16.98)	88 (28.03)	0.025	0.59 (0.36–0.95)	0.077	0.62 (0.35–1.07)	0.09	0.52 (0.21–1.15)
*A*26:01* N India	41 (6.03)	32 (6.45)	9 (4.89)	69 (10.55)	0.0027	0.54 (0.35–0.82)	0.015	0.58 (0.36–0.92)	0.019	0.43 (0.18–0.90)
Gujarat	11 (6.47)	8 (6.84)	3 (5.66)	37 (11.78)	0.061	0.51 (0.23–1.07)	0.13	0.54 (0.21–1.24)	0.13[1]	0.51 (0.22–1.14)[2]
*A*31:01* N India	24 (3.53)	17 (3.43)	7 (3.80)	50 (7.65)	0.001	0.44 (0.25–0.74)	0.0025	0.42 (0.22–0.76)	0.06	0.47 (0.18–1.08)
Gujarat	9 (5.29)	7 (5.98)	2 (3.77)	16 (5.10)	0.92	1.04 (0.39–2.56)	0.71	1.18 (0.40–3.14)	0.50[1]	0.87 (0.33–2.29)[2]
*A*33:01* N India	94 (13.82)	59 (11.90)	35 (19.02)	71 (10.86)	0.09	1.31 (0.93–1.85)	0.58	1.10 (0.75–1.62)	0.003	1.92 (1.19–3.05)
Gujarat	20 (11.76)	15 (12.82)	5 (9.43)	34 (10.83)	0.75	1.09 (0.57–2.04)	0.56	1.21 (0.58–2.39)	0.49[1]	0.92 (0.47–1.80)[2]
*A*68:01* N India	107 (15.74)	87 (17.54)	20 (10.87)	100 (15.29)	0.82	1.03 (0.76–1.40)	0.30	1.17 (0.84–1.63)	0.13	0.67 (0.38–1.14)
Gujarat	44 (25.88)	32 (27.35)	12 (22.64)	38 (12.10)	0.0001	2.53 (1.52–4.23)	0.0001	2.73 (1.54–4.79)	0.038	2.12 (0.93–4.57)
HLA-B										
*B*08:01* N India	37 (5.44)	30 (6.05)	7 (3.80)	96 (14.68)	1.80×10^{-8}	0.33 (0.21–0.50)	3.47×10^{-6}	0.37 (023–0.58)	0.00007	0.22 (0.08–0.50)
Gujarat	8 (4.71)	4 (3.45)	4 (3.45)	32 (10.32)	0.033	0.42 (0.16–0.98)	0.014[1]	0.34 (0.16–0.70)[2]	0.35[1]	0.76 (0.37–1.58)[2]
*B*13:01* N India	40 (5.88)	30 (6.05)	10 (5.43)	15 (2.29)	0.00098	2.66 (1.42–5.23)	0.001	2.74 (1.41–5.54)	0.02	2.44 (0.96–5.92)
Gujarat	5 (2.94)	3 (2.59)	2 (1.72)	2 (0.65)	0.057[1]	4.10 (1.41–11.90)[2]	0.12[1]	3.80 (1.19–12.11)[2]	0.10[1]	5.87 (1.67–20.64)[2]
*B*15:02* N India	39 (5.74)	29 (5.85)	10 (5.43)	16 (2.45)	0.002	2.42 (1.30–4.9)	0.003	2.47 (1.28–4.93)	0.038	2.29 (0.91–5.47)
Gujarat	16 (9.41)	13 (11.21)	3 (2.59)	26 (8.39)	0.70	1.13 (0.55–2.27)	0.34	1.39 (0.63–2.94)	0.34[1]	0.72 (0.32–1.65)[2]
*B*37:01* N India	70 (10.29)	53 (10.69)	17 (9.24)	29 (4.43)	0.00004	2.47 (1.55–4.01)	0.00004	2.57 (1.57–4.27)	0.01	2.19 (1.10–4.24)
Gujarat	29 (17.06)	19 (16.38)	10 (8.62)	23 (7.42)	0.001	2.56 (1.37–4.82)	0.0057	2.44 (1.19–4.90)	0.008	2.83 (1.12–6.68)
*B*44:03* N India	39 (5.74)	24 (4.84)	15 (8.15)	18 (2.75)	0.007	2.14 (1.18–4.03)	0.06	1.79 (0.92–3.55)	0.00087	3.13 (1.43–6.73)
Gujarat	4 (2.35)	3 (2.59)	1 (0.8)	14 (4.52)	0.17[1]	0.55 (0.26–1.18)[2]	0.27[1]	0.63 (0.27–1.45)[2]	0.31[1]	0.57 (0.17–1.91)[2]
*B*51:01* N India	93 (13.68)	79 (15.93)	14 (7.61)	138 (21.10)	0.0003	0.59 (0.43–0.79)	0.026	0.70 (0.51–0.97)	0.000027	0.30 (0.16–0.55)
Gujarat	23 (13.53)	16 (13.79)	7 (6.03)	42 (13.55)	0.99	0.99 (0.55–1.77)	0.94	1.02 (0.51–1.95)	0.90	0.95 (0.33–2.30)
*B*57:01* N India	24 (3.53)	17 (3.43)	7 (3.80)	25 (3.82)	0.77	0.92 (0.49–1.69)	0.72	0.89 (0.44–1.74)	0.99	0.99 (0.35–2.41)
Gujarat	8 (4.71)	3 (2.59)	5 (4.31)	22 (7.10)	0.30	0.64 (0.24–1.55)	0.055[1]	0.39 (0.17–0.89)[2]	0.37[1]	1.42 (0.71–2.84)[2]
*B*58:01* N India	39 (5.74)	24 (4.84)	15 (8.15)	59 (9.02)	0.02	0.61 (0.39–0.95)	0.006	0.51 (0.30–0.85)	0.71	0.89 (0.45–1.64)
Gujarat	7 (4.12)	3 (2.59)	4 (3.45)	16 (5.16)	0.60	0.78 (0.26–2.08)	0.19[1]	0.55 (0.24–1.26)[2]	0.34[1]	1.59 (0.74–3.42)[2]
HLA-DRB1										
*DRB1*01:01* N India	35 (5.15)	25 (5.04)	10 (5.43)	35 (5.35)	0.86	0.95 (0.57–1.60)	0.81	0.93 (0.53–1.63)	0.96	1.01 (0.44–2.15)
Gujarat	4 (2.34)	2 (1.71)	2 (3.70)	25 (7.58)	0.01[1]	0.32 (0.15–0.66)[2]	0.01[1]	0.25 (0.10–0.65)[2]	0.23[1]	0.57 (0.22–1.46)[2]
*DRB1*03:01* N India	65 (9.56)	49 (9.88)	16 (8.70)	155 (23.70)	3.46×10^{-12}	0.34 (0.24–0.46)	1.22×10^{-9}	0.35 (0.24–0.50)	8.13×10^{-6}	0.30 (0.16–0.53)
Gujarat	12 (7.02)	7 (5.98)	5 (9.26)	60 (18.18)	0.0007	0.33 (0.16–0.66)	0.0015	0.28 (0.10–0.65)	0.071[1]	0.49 (0.26–0.95)[2]
*DRB1*04:03* N India	65 (9.56)	46 (9.27)	19 (10.33)	47 (7.19)	0.11	1.36 (0.90–2.06)	0.19	1.32 (0.84–2.06)[2]	0.16	1.48 (0.80–2.66)
Gujarat	2 (1.17)	0 (0.00)	2 (3.70)	24 (7.27)	0.0016[1]	0.18 (0.07–0.46)[2]	0.0005[1]	0.05 (0.007–0.38)[2]	0.26[1]	0.59 (0.23–1.52)[2]
*DRB1*10:01* N India	105 (15.44)	81 (16.33)	24 (13.04)	64 (9.79)	0.0019	1.68 (1.19–2.38)	0.0009	1.79 (1.24–2.59)	0.20	1.38 (0.80–2.32)
Gujarat	40 (23.39)	26 (22.22)	14 (25.93)	39 (11.82)	0.0007	2.27 (1.35–3.81)	0.006	2.13 (1.17–3.81)	0.005	2.61 (1.19–5.43)
*DRB1*11:01* N India	73 (10.74)	56 (11.29)	17 (9.24)	77 (11.77)	0.55	0.90 (0.63–1.28)	0.79	0.95 (0.64–1.39)	0.33	0.76 (0.41–1.34)
Gujarat	20 (11.70)	17 (14.53)	3 (5.56)	41 (12.42)	0.81	0.93 (0.49–1.69)	0.56	1.19 (0.60–2.27)	0.10[1]	0.47 (0.21–1.05)[2]
*DRB1*12:02* N India	52 (7.65)	41 (8.27)	11 (5.98)	12 (1.83)	6.84×10^{-7}	4.42 (2.30–9.19)	2.58×10^{-7}	4.82 (2.44–10.17)	0.002	3.40 (1.33–8.56)

Table 3 continued on following page

Table 3. Continued

	Vitiligo	Generalized vitiligo	Localized vitiligo	Controls	Vitiligo versus controls		Generalized vitiligo versus controls		Localized vitiligo versus controls	
HLA allele	No. (%)	No. (%)	No. (%)	No. (%)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
Gujarat	12 (7.02)	10 (8.55)	2 (3.70)	22 (6.67)	0.88	1.05 (0.46–2.29)	0.49	1.30 (0.53–2.98)	0.31[1]	0.65 (0.25–1.68)[2]
*DRB1*14:04* N India	92 (13.53)	62 (12.50)	30 (16.30)	56 (8.56)	0.0039	1.67 (1.16–2.41)	0.029	1.52 (1.02–2.27)	0.002	2.08 (1.24–3.42)
Gujarat	22 (12.87)	16 (13.68)	6 (11.11)	39 (11.82)	0.73	1.10 (0.59–1.98)	0.13	1.51 (0.83–2.68)	0.88	0.93 (0.30–2.38)

Abbreviations: CI, confidence interval; N, North; OR, odds ratio.
[1]*P*-value was calculated by using Fisher's exact test.
[2]OR was calculated by using Woolf's method with Haldane's modification.
All patient and control samples with *DRB1*07:01* allele were removed to study the contribution of alleles independent of *DRB1*07:01* in susceptibility to vitiligo. All vitiligo (*N*=680), generalized (*N*=496), and localized (*N*=184) vitiligo samples from North India were compared with healthy controls (*N*=654) for *HLA-A*, *HLA-B*, and *HLA-DRB1* loci. From Gujarat, all vitiligo (*N*=170), generalized (*N*=117), and localized (*N*=53) vitiligo samples were compared with 314 unaffected controls for *HLA-A* locus. Overall, 170 vitiligo samples, 116 generalized and 54 localized, were compared with 314 samples from unaffected controls for *HLA-B* locus, and 171 vitiligo samples, 117 generalized and 54 localized, were compared with 330 unaffected controls for *HLA-DRB1* locus.

the initial study in both generalized and localized vitiligo. However, only *B*44:03* and *DRB1*07:01* were associated in the replication study with both the generalized and localized types of vitiligo (Table 2 and Supplementary Table S1 online). Alleles associated with different manifestations of vitiligo were *HLA-A*02:01*, *B*37:01*, *B*57:01*, *DRB1*04:03*, and *DRB1*10:01*, which were significantly increased, and *A*31:01*, *B*58:01*, *C*04:01*, *DRB1*01:01*, *DRB1*11:01*, and *DRB1*15:02* were significantly reduced in generalized vitiligo cases and not in localized vitiligo cases from North India. *HLA-A*30:01*, *A*33:01*, and *A*68:01* were significantly increased, and *B*58:01* and *DRB1*01:01* were significantly reduced in generalized vitiligo cases and not in localized vitiligo cases from Gujarat as compared with unaffected controls.

In the absence of *DRB1*07:01*, *B*37:01* was significantly increased in both North Indian and Gujarati patients, irrespective of the type of vitiligo. In addition, *A*68:01* and *DRB1*10:01* were significantly increased in patients from Gujarat and *B*13:01*, *B*15:02*, *DRB1*12:02*, and *DRB1*14:04* were significantly increased in North Indian patients, irrespective of the type of vitiligo. *A*33:01* and *B*44:03* were increased in localized cases and *DRB1*10:01* was increased in generalized cases from North India (Table 3).

Haplotype *A*33:01-B*44:03-DRB1*07:01* (Table 4) was significantly increased in both generalized and localized cases in the initial study and only in generalized cases in the replication study. *A*24:02-B*44:03-DRB1*07:01* was significantly increased in generalized cases from North India, whereas both generalized and localized cases from Gujarat showed a significant increase in the frequency of this haplotype when compared with unaffected controls. Haplotype *A*02:01-B*44:03-DRB1*07:01* was significantly increased in both generalized and localized vitiligo cases from North India.

Two-locus haplotype analysis showed that *B*44:03-DRB1*07:01* was significantly increased in both generalized and localized cases in both initial and replication studies (Table 5). *A*33:01-DRB1*07:01* and *A*02:01-DRB1*07:01* were significantly increased in both generalized and localized vitiligo cases in the initial study; however, only haplotype *A*33:01-DRB1*07:01* was significantly increased in generalized vitiligo cases from Gujarat. Haplotype *A*26:01-DRB1*03:01* was significantly reduced in both generalized and localized cases from North India and Gujarat, although *P*-value did not reach statistical significance for localized cases from Gujarat because of smaller sample size. *B*08:01-DRB1*03:01* was significantly reduced in both generalized and localized vitiligo cases from North India and Gujarat.

Specific amino-acid signatures of HLA peptide-binding pockets predispose to vitiligo

Amino-acid signatures of the peptide-binding pockets of HLA-A, -B, and -DRB1 were compared in vitiligo cases with unaffected controls (Figure 1 and Supplementary Tables S9 and S10 online). For HLA-A and -B, five key residues constituting the B pocket (positions 9, 45, 63, 66, and 67) and four key residues constituting the F pocket (positions 77, 80, 81, and 116) of the α1 and α2 domains of the α-chain were investigated (Sidney *et al.*, 2008). For DRB1, 11 residues representing integral parts of peptide-binding pockets of DR β-chain were analyzed at positions 26, 28, 30, 37, 47, 67, 70, 71, 74, 77, and 86 (Menconi *et al.*, 2010). The protein sequences were downloaded from the HLA database (http://www.ebi.ac.uk/imgt/hla/align.html), and the amino acids present at the aforesaid positions were compared in patients and controls. Approximately 8–10% of the samples were homozygous for one or more loci in patients and controls. Thus, to avoid overrepresentation of certain amino acids at particular positions because of homozygosity, we considered phenotype frequencies of the alleles; i.e., the homozygous samples were counted as having only one allele. The results show that that $Glu^{28\beta}$, $Leu^{30\beta}$/$Arg^{30\beta}$ $Phe^{37\beta}$, $Tyr^{47\beta}$, $Ile^{67\beta}$, $Asp^{70\beta}$, $Arg^{71\beta}$, $Gln^{74\beta}$, $Thr^{77\beta}$, and $Gly^{86\beta}$ make the molecular signature of the peptide-binding pockets of DRB1 for predisposition to develop vitiligo in both the initial and replication studies (Figure 1). $Asp^{28\beta}$, $Tyr^{30\beta}$/$Cys^{30\beta}$, $Ser^{37\beta}$, $Phe^{47\beta}$, $Leu^{67\beta}$, $Gln^{70\beta}$, $Lys^{71\beta}$, Ala/$Arg^{74\beta}$, $Asn^{77\beta}$, and $Val^{86\beta}$

Table 4. *HLA-A-B-DRB1* (three-locus) haplotypes significantly different in vitiligo cases as compared with controls from North India and Gujarat

		North India vitiligo versus North India controls			Gujarat vitiligo versus Gujarat controls		
HLA haplotype	Sample type	No. (%)	*P*-value	OR (95% CI)	No. (%)	*P*-value	OR (95% CI)
*A*33:01-B*44:03-DRB1*07:01*	Controls	27 (2.99)			14 (3.57)		
	All cases	226 (16.09)	8.75×10^{-23}	6.21 (4.01–9.72)	42 (11.93)	0.000016	3.65 (1.91–7.37)
	Generalized	189 (17.22)	1.94×10^{-24}	6.74 (4.43–10.60)	34 (13.70)	2.09×10^{-6}	4.28 (2.18–8.83)
	Localized	37 (12.05)	9.18×10^{-10}	4.44 (2.57–7.72)	8 (7.69)	0.07	2.25 (0.79–5.93)
*A*02:01-B*44:03-DRB1*07:01*	Controls	0 (0.00)			0 (0.00)		
	All cases	33 (2.35)	6.66×10^{-8} [1]	44.08 (6.11–317.86)[2]	1 (0.28)	0.47[1]	3.34 (0.34–32.27)[2]
	Generalized	29 (2.64)	2.33×10^{-8} [1]	49.83 (6.83–360.03)[2]	0 (0.00)		
	Localized	4 (1.30)	0.004[1]	26.76 (3.38–211.67)[2]	1 (0.96)	0.20[1]	11.37 (1.17–109.93)[2]
*A*24:02-B*44:03-DRB1*07:01*	Controls	4 (0.44)			2 (0.51)		
	All cases	26 (1.85)	0.0019[1]	3.83 (1.88–7.80)[2]	12 (3.40)	0.0034[1]	5.73 (2.18–15.06)[2]
	Generalized	23 (2.09)	0.0009[1]	4.37 (2.13–8.93)[2]	7 (2.82)	0.020[1]	4.85 (1.75–13.43)[2]
	Localized	3 (0.97)	0.25[1]	2.29 (0.85–6.18)[2]	5 (4.80)	0.005[1]	8.63 (2.96–25.13)[2]
*A*26:01-B*08:01-DRB1*03:01*	Controls	37 (4.10)			17 (4.33)		
	All cases	20 (1.42)	0.00005	0.33 (0.18–0.60)	2 (0.56)	0.0007[1]	0.15 (0.05–0.39)[2]
	Generalized	16 (1.45)	0.00025	0.34 (0.18–0.64)	1 (0.40)	0.0016[1]	0.13 (0.04–0.42)[2]
	Localized	4 (1.30)	0.010[1]	0.34 (0.17–0.68)[2]	1 (0.96)	0.08[1]	0.31 (0.09–1.02)[2]
*A*01:01-B*57:01-DRB1*07:01*	Controls	30 (3.32)			27 (6.88)		
	All cases	81 (5.76)	0.0075	1.77 (1.42–2.82)	27 (7.67)	0.68	1.12 (0.61–2.03)
	Generalized	65 (5.92)	0.0065	1.83 (1.15–2.95)	22 (8.87)	0.35	1.31 (0.70–2.46)
	Localized	16 (5.21)	0.13	1.59 (0.80–3.08)	5 (4.80)	0.30[1]	0.73 (0.37–1.43)[2]
*A*11:01-B*44:03-DRB1*07:01*	Controls	16 (1.77)			1 (0.25)		
	All cases	43 (3.06)	0.055	1.74 (0.95–3.34)	9 (2.55)	0.006[1]	7.21 (2.12–24.49)[2]
	Generalized	36 (3.28)	0.035	1.87 (1.00–3.65)	7 (2.82)	0.006[1]	8.1 (2.33–28.14)[2]
	Localized	7 (2.28)	0.57	1.29 (0.44–3.36)	2 (1.92)	0.11[1]	6.36 (1.50–26.85)[2]
*A*11:01-B*57:01-DRB1*07:01*	Controls	8 (0.99)			15 (3.82)		
	All cases	14 (0.99)	0.79	1.12 (0.43–3.10)	5 (1.42)	0.034[1]	0.38 (0.19–0.77)[2]
	Generalized	12 (1.09)	0.64	1.23 (0.46–3.50)	3 (1.20)	0.04[1]	0.34 (0.15–0.79)[2]
	Localized	2 (0.65)	0.51[1]	1.69 (0.54–4.91)[2]	2 (1.92)	0.27[1]	0.59 (0.22–1.54)[2]

Abbreviations: CI, confidence interval; OR, odds ratio.
[1]*P*-value was calculated by using Fisher's exact test.
[2]OR was calculated by using Woolf's method with Haldane's modification.
Three-locus haplotypes were constructed using Arlequin ver 3.5 for 1,404 vitiligo patients referred to as all cases in the table (1,097 generalized and 307 localized vitiligo cases) from North India and 352 vitiligo patients from Gujarat (248 generalized and 104 localized vitiligo cases) and compared with 902 and 392 unaffected controls from North India and Gujarat, respectively.

make the molecular signature of DRB1 for the protection from vitiligo in both North India and Gujarat. The molecular signature for HLA-A alleles for predisposition to develop the disease is actually specific for HLA-A allele *A*33:01*, which is in LD with *DRB1*07:01*. For HLA-B locus, $Lys^{45\alpha}$, $Glu^{63\alpha}$, $Ser^{67\alpha}$, $Asn^{77\alpha}$, $Thr^{80\alpha}$, $Ala^{81\alpha}$, and $Ser^{99\alpha}$ makes the molecular signature for predisposition to develop vitiligo, and $Thr^{45\alpha}$, $Asn^{63\alpha}$, $Phe^{67\alpha}$, $Ser^{77\alpha}$, $Asn^{80\alpha}$, $Leu^{81\alpha}$, and $Tyr^{99\alpha}$ makes the molecular signature for protection from vitiligo in both the initial and replication studes. In addition, significant increase of $Met^{45\alpha}$, $Cys^{67\alpha}$, $Asp^{77\alpha}$, and $Asp^{116\alpha}$ in North Indian samples suggests the involvement of alleles other than

Table 5. *HLA-A-DRB1* and *HLA-B-DRB1* (two-locus) haplotypes showing significant differences in vitiligo patients as compared with controls from North India and Gujarat

		North India vitiligo versus North India controls			Gujarat vitiligo versus Gujarat controls		
HLA haplotype	Sample type	No. (%)	*P*-value	OR (95% CI)	No. (%)	*P*-value	OR (95% CI)
*A*33:01-DRB1*07:01*	Controls	29 (7.39)			15 (3.82)		
	All cases	251 (17.87)	6.97×10^{-26}	6.55 (4.39–10.08)	44 (12.50)	0.00001	3.59 (1.91–7.07)
	Generalized	212 (19.32)	3.50×10^{-28}	7.21 (4.81–11.14)	36 (14.51)	1.44×10^{-6}	4.26 (2.21–8.57)
	Localized	39 (12.70)	4.58×10^{-10}	4.38 (2.58–7.48)	8 (7.69)	0.09	2.09 (0.74–5.43)
*A*02:01-DRB1*07:01*	Controls	7 (1.10)			0 (0.00)		
	All cases	66 (4.70)	1.49×10^{-7}	6.30 (2.87–16.35)	3 (0.85)	0.10[1]	7.86 (0.96–64.05)[2]
	Generalized	56 (5.10)	3.53×10^{-8}	6.87 (3.10–17.96)	0 (0.00)		
	Localized	10 (3.25)	0.001	4.30 (1.46–13.43)	3 (2.88)	0.009[1]	27.06 (3.31–221.26)[2]
*B*44:03-DRB1*07:01*	Controls	82 (9.09)			19 (4.84)		
	All cases	402 (28.63)	2.43×10^{-29}	4.01 (3.09–5.23)	86 (24.43)	1.84×10^{-14}	6.34 (3.71–11.30)
	Generalized	343 (31.26)	1.74×10^{-33}	4.54 (3.48–5.97)	63 (25.40)	3.45×10^{-14}	6.68 (3.80–12.15)
	Localized	59 (19.21)	1.79×10^{-6}	2.37 (1.62–3.47)	23 (22.11)	1.87×10^{-8}	5.57 (2.74–11.33)
*B*57:01-DRB1*07:01*	Controls	47 (5.21)			45 (4.98)		
	All cases	128 (9.11)	0.0005	1.82 (1.28–2.63)	44 (12.50)	0.66	1.10 (0.69–1.75)
	Generalized	103 (9.38)	0.0004	1.88 (1.30–2.75)	35 (14.11)	0.32	1.26 (0.76–2.08)
	Localized	25 (8.14)	0.60	1.61 (0.93–2.73)	9 (8.65)	0.41	0.73 (0.30–1.58)
*A*24:02-DRB1*12:02*	Controls	3 (0.33)			13 (3.31)		
	All cases	12 (0.85)	0.10[1]	2.30 (0.99–5.34)[2]	3 (0.85)	0.01[1]	0.28 (0.12–0.65)[2]
	Generalized	8 (0.72)	0.18[1]	2.00 (0.82–4.84)[2]	3 (1.20)	0.076[1]	0.40 (0.17–0.92)[2]
	Localized	4 (1.30)	0.07[1]	3.81 (1.41–10.27)[2]	0 (0.00)	0.045[1]	0.13 (0.02–0.99)[2]
*A*26:01-DRB1*03:01*	Controls	37 (4.10)			17 (4.33)		
	All cases	20 (1.42)	0.00005	0.33 (0.18–0.60)	2 (0.56)	0.0007[1]	0.15 (0.05–0.39)[2]
	Generalized	16 (1.45)	0.00025	0.34 (0.18–0.64)	1 (0.40)	0.0016[1]	0.13 (0.04–0.42)[2]
	Localized	4 (1.30)	0.01[1]	0.34 (0.17–0.69)[2]	1 (0.96)	0.07[1]	0.31 (0.09–1.02)[2]
*B*08:01-DRB1*03:01*	Controls	71 (7.87)			27 (6.88)		
	All cases	24 (1.70)	3.70×10^{-13}	0.20 (0.12–0.33)	4 (1.61)	0.00004[1]	0.17 (0.08–0.34)[2]
	Generalized	18 (1.64)	1.80×10^{-11}	0.19 (0.11–0.33)	3 (1.20)	0.0004[1]	0.18 (0.08–0.42)[2]
	Localized	6 (1.95)	0.0002	0.23 (0.08–0.54)	1 (0.96)	0.01[1]	0.19 (0.06–0.62)[2]

Abbreviations: CI, confidence interval; OR, odds ratio.
[1]*P*-value was calculated by using Fisher's exact test.
[2]OR was calculated by using Woolf's method with Haldane's modification.
Two-locus haplotypes (*HLA-A-HLA-DRB1* and *HLA-B-HLA-DRB1*) were deduced from three-locus haplotypes (Arlequin ver 3.5) for patients and controls as described in Table 4.

the predisposing alleles, which may be in low frequencies (Figure 1, Supplementary Table S10 online).

Subtle differences were observed in the molecular signatures of the peptide-binding pockets of DR β-chain in localized and generalized vitiligo. Interestingly, localized vitiligo patients show similarities in the amino-acid signatures with generalized vitiligo at positions $Glu^{28\beta}$, $Leu^{30\beta}$, $Phe^{37\beta}$, $Tyr^{47\beta}$, and $Gln^{74\beta}$ in both the initial and replication studies. However, they show similarities with unaffected controls at positions $Arg^{30\beta}$/$Cys^{30\beta}$, $Ser^{37\beta}$, $Ile^{67\beta}$/$Leu^{67\beta}$ $Asp^{70\beta}$, and

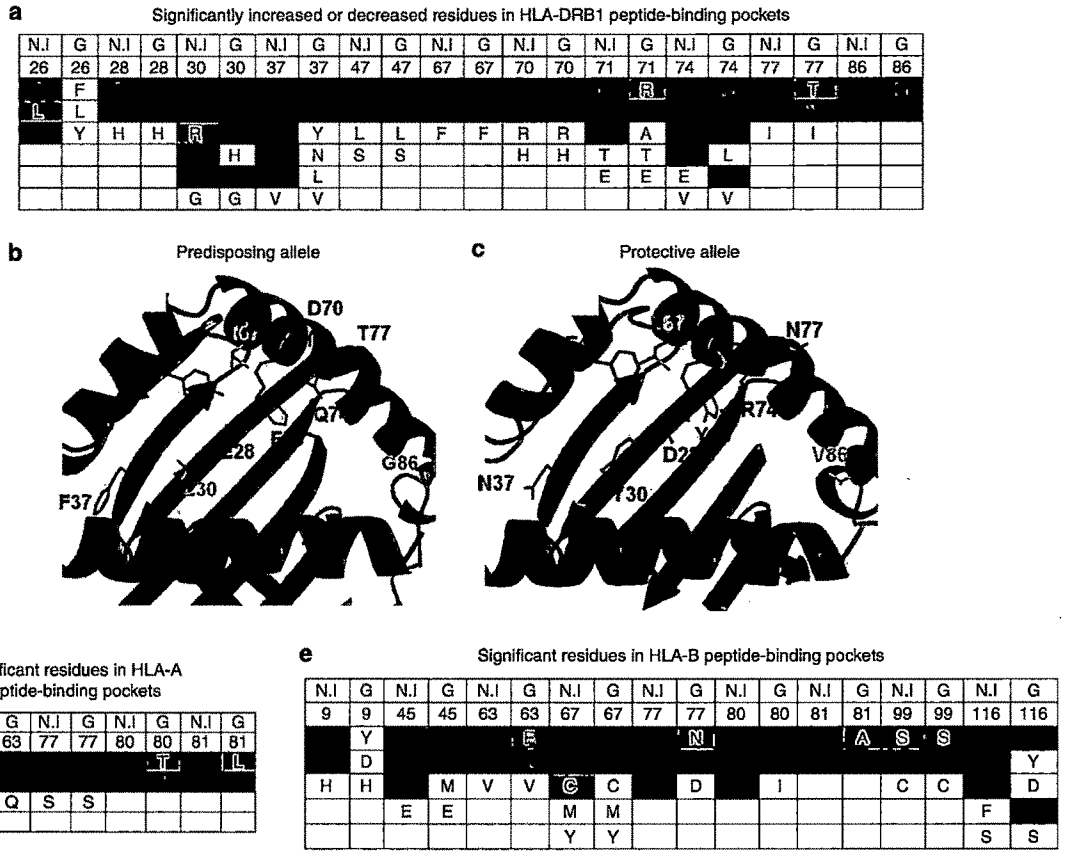

Figure 1. Amino-acid signatures for the peptide-binding pockets of HLA molecules. (**a**) For DR β1-chain in vitiligo patients from North India and Gujarat. Amino acids shown in red are significantly increased in vitiligo patients; amino acids shown in green are significantly reduced in vitiligo patients as compared with controls. More than one amino acid at certain positions being red or green suggests the involvement of multiples alleles. (**b**) Ribbon diagram for peptide-binding clefts for predisposing and (**c**) protective DRB1 alleles (using PyMOL software, PyMOL Molecular Graphics System, Schrödinger, San Carlos, CA). (**d**) Amino-acid signature for HLA-A locus. (**e**) Amino-acid signature for HLA-B locus in vitiligo. ■ Significantly increased $P<0.009$ to 10^{-29}; ■ Significantly increased $P<0.05$–0.01; ■ Significantly reduced $P<0.009$ to 10^{-28}; ■ Significantly reduced $P<0.05$–0.01; □ Nonsignificant.

$Ala^{74\beta}$ in North Indians, and $Cys^{30\beta}$, $Ser^{37\beta}$, $Asp^{70\beta}$, $Arg^{71\beta}$/Lys $Ser^{71\beta}$, $Ala^{74\beta}$/$Arg^{74\beta}$, and $Thr^{77\beta}$/$Asn^{77\beta}$ in Gujaratis (data not shown).

DISCUSSION

Our results show that *A*33:01*, *B*44:03*, and *DRB1*07:01* were significantly increased in both initial and replication studies, suggesting that these alleles are the markers for vitiligo in both North India and Gujarat. Association of *DRB1*07:01* with vitiligo seems to be primary, because in the absence of *A*33:01*, *B*44:03*, or *B*37:01*, *DRB1*07:01* still remains highly significant in both the populations studied, whereas the converse was not true. The basic predisposing alleles in both localized and generalized vitiligo are the same; however, similarities with the controls in terms of allele frequencies of some alleles and amino-acid signature of the DR β-chain seem to be protective from generalized distribution of the lesions in localized vitiligo (Supplementary Table S11 online).

Haplotype analysis showed the presence of 177 and 68 *DRB1*07:01* haplotypes in North Indian and Gujarati cases, respectively, suggesting that enough recombinational events have taken place during the course of evolution, giving rise to such a diversity of haplotypes; however, some of the predisposing and protective haplotypes are still conserved. The haplotype *A*33:01-B*44:03-DRB1*07:01* was significantly increased in generalized and localized vitiligo cases from North India and only generalized cases from Gujarat. Although the localized cases from Gujarat showed an OR of 2.25 for this haplotype, the *P*-value was not significant, probably because of smaller sample size for the localized cases from Gujarat.

Very few studies have been conducted on association of HLA alleles with vitiligo using low-resolution molecular methods and still fewer using high-resolution typing methods. A study on 41 Turkish vitiligo cases showed *DRB1*03*, *DRB1*04*, and *DRB1*07* to be increased in the vitiligo cases (Tastan *et al.*, 2004). However, we observed *DRB1*07:01* to

be positively associated and *DRB1*03:01* to be negatively associated with vitiligo in both initial and replication studies. Presence of *DRB1*07:01* has been reported in patients from Slovakia (Buc *et al.*, 1998) and China (Ren *et al.*, 2009), whereas HLA class I alleles have not been reported in these studies. A meta-analysis of 11 case-control studies ($N=777$) showed that class I allele HLA-A2 is associated with vitiligo (Liu *et al.*, 2007). HLA-A2 is a cluster of 266 alleles, one of which, *HLA-A*02:01*, was significantly increased in generalized vitiligo cases ($P<0.001$, OR = 1.57) from North India, although only 14.59% of the generalized vitiligo patients and 9.76% of unaffected controls had this allele (Table 2). The haplotype *A*02:01-B*44:03-DRB1*07:01* was significantly increased in both generalized (2.64%) and localized cases (1.3%) from North India as compared with controls who lacked this haplotype altogether. However, our data suggest that the primary association of *HLA* with vitiligo in North India and Gujarat is not with *A*02:01*, but with A*33:01, *B*44:03*, and *DRB1*07:01*.

Analysis of two-locus haplotypes for *HLA-A-DRB1* and *HLA-B-DRB1* suggests that the haplotype *B*44:03-DRB1*07:01* is the marker for both generalized and localized vitiligo in both the populations studied. *DRB1*07:01* was also in LD with *A*02:01*, *A*33:01*, and *B*57:01*. These data suggest that the autoreactive $CD4^+$ T-helper cells are restricted by *HLA-DRB1*07:01* and the autoreactive $CD8^+$ cytotoxic T cells may be restricted by *HLA-A*33:01*, *A*02:01*, *B*44:03*, and *B*57:01* in the Indian populations studied.

Association of *MHC* alleles with a disease gains importance because of the antigen-presenting function of the MHC. The peptides presented by the MHC molecules have allele-specific motifs (Falk *et al.*, 1991). The affinity of the peptide to a particular MHC molecule is determined by the amino-acid residues present in peptide-binding pockets of the peptide-binding groove. Shared amino acids in the peptide-binding pockets have been demonstrated in autoimmune diseases such as Type 1 diabetes (Todd *et al.*, 1987), rheumatoid arthritis (Winchester, 1994), and thyroiditis (Menconi *et al.*, 2010). Investigation of amino-acid signatures for the peptide-binding pockets of HLA-A and HLA-B α-chain and HLA-DR β-chain revealed specific molecular signatures for predisposition and protection from the disease. These results suggest affinity of autoantigenic peptides for predisposing MHC class II and class I molecules that may be involved in orchestrating (through $CD4^+$ T cells) and implementing the autoimmune responses (through $CD8^+$ T cells) in vitiligo, respectively.

Generalized vitiligo is considered to be an autoimmune disease, whereas localized vitiligo has not been considered an autoimmune disease. We observed *B*44:03* and *DRB1*07:01* to be predisposing in both generalized and localized vitiligo patients in both the populations studied, beside some differences in the frequencies of some other alleles. To our knowledge, this is previously unreported. Association of *MHC* alleles with localized vitiligo clearly shows that there is a need to relook at the etiopathogenesis of localized vitiligo. Although it may be an autoimmune disorder, similarities with unaffected controls in terms of *HLA* alleles and amino-acid signatures of the peptide-binding pockets of DR β-chain may be contributing to the localized distribution of the lesions.

MATERIALS AND METHODS

Patient group

DNAs were extracted from 1,404 North Indian patients with vitiligo enrolled at Dr Ram Manohar Lohia Hospital and All India Institute of Medical Sciences, New Delhi, after obtaining informed consent. These subjects belonged to North Indian states of Uttar Pradesh, Himachal Pradesh, Bihar, Haryana, Punjab, and Delhi. All cases and control samples were collected after Institutional Human Ethics Committee's clearance from all the institutes/hospitals involved, following the Declaration of Helsinki Principles. Diagnosis of vitiligo was based on clinical examination done by dermatologists. Clinically, the cases were classified as having generalized (vulgaris, acrofacial, and universalis) or localized (focal, acral, mucosal, and segmental) forms of vitiligo. The replication study was carried out on 355 vitiligo cases from Gujarat, a state in west of India (Table 1).

It may be pertinent to mention here that the two groups studied in the initial and replication studies form two independent endogamous groups that do not inter-marry.

Unaffected controls

A total of 902 ethnically matched unaffected controls from North India were used in the initial study and 441 ethnically matched unaffected controls from Gujarat were studied in the replication study. None of the unaffected controls had personal or family history of vitiligo or any other autoimmune disease. Of the 902 controls from North India, blood samples of 308 were collected at National Institute of Immunology, Dr Ram Manohar Lohia Hospital and All India Institute of Medical Sciences, New Delhi, and the remaining 594 samples were procured from the Institute of Genomics and Integrative Biology, New Delhi, which were collected from the aforesaid states of North India as a part of the Indian Genome Variation program, and the details of the sample collection have been explained elsewhere (Consortium, 2005).

Study of *HLA-A, HLA-B, HLA-C,* and *HLA-DRB1* alleles

DNA extraction was carried out by standard procedures from blood samples collected in anticoagulant EDTA. Alleles of *HLA-A, HLA-B, HLA-C,* and *HLA-DRB1* were studied using PCR, followed by hybridization with sequence-specific oligonucleotide probes as described earlier (Israni *et al.*, 2009), using a bead-based technology (Luminex, Austin, TX) following the manufacturer's instructions (Labtype SSO kit from One Lambda, Canoga Park, LA). All North Indian samples were typed for *HLA-A, HLA-B,* and *HLA-DRB1* loci. A total of 404 vitiligo and 438 unaffected control samples from North India were typed for HLA-C locus. Of the 355 Gujarat cases, 354, 353, and 355 cases were typed for *HLA-A, HLA-B,* and *HLA-DRB1* loci, respectively, and of the 441 unaffected controls from Gujarat population, 408, 407, and 441 individuals were typed for three loci, respectively. Differences in the sample numbers studied for different loci are because of amplification failures in some samples. The latest nomenclature for the *HLA* system was used to designate the alleles of the three loci studied (Marsh *et al.*, 2010).

Haplotype analysis

Estimation of *HLA* haplotype frequencies was carried out using the expectation maximization algorithm (Excoffier and Slatkin, 1995; Excoffier *et al.*, 2005). Arlequin Ver 3.5 (Excoffier *et al.*, 2005) was run using the following settings: expectation maximization algorithm performed at the haplotype level, $\varepsilon = 1e{-}7$, five significant digits for output, 50 starting points for expectation maximization algorithm, and a maximum of 1,000 iterations (Bettencourt *et al.*, 2008) at http://cmpg.unibe.ch/software/arlequin35/. Three- and four-locus haplotypes were thus constructed for both patient and controls samples to identify predisposing and protective haplotypes in the two populations. Two-locus haplotypes were derived from three-locus haplotypes.

Statistical analysis

Vitiligo samples were compared with their respective unaffected controls using χ^2-analysis using Stata 9.2 statistical program. Fisher's exact test was used when the numbers were five or less in any group; i.e., in cases or controls for any allele. In such cases, ORs were calculated using Woolf's method (Woolf, 1955) with Haldane (1956) modification as described earlier (Rani *et al.*, 1998).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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CLINICAL AND LABORATORY INVESTIGATIONS

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Interleukin-4 genetic variants correlate with its transcript and protein levels in patients with vitiligo

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Summary

Background Vitiligo is an acquired pigmentary disorder resulting from loss of melanocytes. Interleukin (IL)-4 has been shown to stimulate B-cell proliferation, to regulate immunoglobulin class switching (IgG1 and IgE) and to promote T-cell development. Polymorphisms in the *IL4* gene are known to increase its expression, thereby implicating its role in vitiligo susceptibility.

Objectives To explore intron 3 VNTR (IVS3) and -590 C/T (rs2243250) promoter polymorphisms in the *IL4* gene and to correlate them with the IL4 transcript, serum IL-4 and IgE levels to achieve genotype–phenotype correlation in patients with vitiligo from Gujarat. A replication study was done in a North Indian population.

Methods The case–control study was performed to investigate these polymorphisms in 505 patients and 744 controls in Gujarat, and 596 patients and 397 controls in North India by polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism analysis. IL4 transcript levels were monitored by real-time PCR. Serum IL-4 and IgE levels were measured by enzyme-linked immunosorbent assay and electrochemiluminescence immunoassay, respectively.

Results The genotype frequencies differed significantly between patients with generalized vitiligo and controls for both the polymorphisms in both populations. Allele frequencies significantly differed between patients with generalized vitiligo and controls for both the polymorphisms in the population from Gujarat. Interestingly, genotype and allele frequencies for -590 C/T single nucleotide polymorphism were significantly different between patients with localized vitiligo and controls in both the populations. The study revealed significantly increased *IL4* mRNA, serum IL-4 and IgE levels in patients from Gujarat. Age of onset analysis of disease in patients suggested that the TTR2R2, TTR1R2 and CTR2R2 haplotypes had a profound effect in the early onset of the disease.

Conclusions Our results suggest that these polymorphisms of the *IL4* gene may be genetic risk factors for susceptibility towards vitiligo and the upregulation of the IL4 transcript, protein and IgE levels in individuals with susceptible haplotypes reveal the crucial role of IL-4 in the pathogenesis of vitiligo.

Vitiligo is a skin disorder with progressive depigmentation of the skin.[1] Absence of melanocytes from the lesional skin due to their destruction has been suggested to be the key event in the pathogenesis of vitiligo.[2] The abnormal immune response frequently observed in patients with vitiligo has led to the suggestion that the condition has an autoimmune component.[3] The autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity.[4,5]

Recently, a number of genes that play a role in vitiligo susceptibility, including *HLA* (human leucocyte antigen), *PTPN22* (protein tyrosine phosphatase, nonreceptor type 22), *NLRP1* (previously *NALP1*; NLR family, pyrin domain containing 1), *XBP1* (X-box binding protein 1), *FOXP1* (forkhead box P1), *IL2RA* [interleukin (IL)-2 receptor, α] have been tested for genetic association with vitiligo.[6]

Cytokines, including interleukins, are important mediators of immunity; hence any imbalance or deficiency in the cytokine

network may lead to autoimmune diseases. Studies indicate that cytokine gene expression is influenced by genetic polymorphisms and these variations appear to be linked with the progression of disease.[7,8] In particular, polymorphisms in the *IL4* gene may have a significant impact on the host immune response. IL-4 is a pleiotropic immunomodulatory cytokine secreted by T-helper (Th) 2 lymphocytes, eosinophils and mast cells.[9] The biological actions of IL-4 include stimulation of IgE and mast cell eosinophil-mediated reactions; IL-4 is the principal cytokine that stimulates B-cell immunoglobulin heavy-chain switching to the IgE isotype, which is the principal mediator of immediate hypersensitivity reactions. Also, IL-4 stimulates the development of Th2 cells from naive CD4+ T cells and functions as an autocrine growth factor for differentiated Th2 cells and also contributes to the maintenance of the Th2 lymphocyte profile that leads to the elevation of baseline IgE levels.[10,11] It has been demonstrated that an imbalance between Th1 and Th2 cytokine production is highly correlated with the induction and development of several autoimmune diseases.[12,13] In particular, *IL4* seems to be an attractive candidate gene on the basis of its key role in IgE production and in the induction of inflammation, thereby contributing towards autoimmunity.

Polymorphisms in the *IL4* gene may cause alterations in its levels, leading to a disturbance in immune functioning, thereby implicating its role in several autoimmune diseases including vitiligo. Genetic variants of the promoter region of *IL4* have been related to elevated levels of serum IgE.[14] The *IL4* promoter region possesses a C to T transition at the -590 position. This polymorphism has been shown to be associated with enhanced promoter strength with increased binding of nuclear transcription factors to the promoter leading to different levels of IL-4 and increased IgG levels against specific antigens.[15,16] Another polymorphism frequently described in the *IL4* gene is a 70-bp VNTR (variable number tandem repeat), located in the intron 3 region. The three tandem repeat allele is known to be a high producer of IL-4.[17]

As IL-4 appears to be an important regulator of the immune response, it is of interest to investigate the role of IL-4 in vitiligo. Hence, the aims of this study were: (i) to determine whether the two well-characterized *IL4* polymorphisms, i.e. IVS3 and -590 C/T single nucleotide polymorphism (SNP), are associated with vitiligo susceptibility in populations from Gujarat and North India; (ii) to measure and compare *IL4* mRNA levels in Gujarati patients with vitiligo and healthy controls with different haplotypes; and (iii) to measure and compare serum IL-4 and IgE levels in Gujarati patients with vitiligo and unaffected controls with different haplotypes.

Materials and methods

Study population

The study population included 505 patients with vitiligo (395 generalized and 110 localized vitiligo cases) from Gujarat and 596 patients with vitiligo (464 generalized and 132 localized vitiligo cases) from North India (Table S1; see Supporting information). A total of 744 ethnically and sex-matched unaffected individuals from Gujarat and 397 individuals from North India were included in the study (Table S1). None of the healthy individuals had any evidence of vitiligo or any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India and the All India Institute of Medical Sciences and National Institute of Immunology, New Delhi, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Determination of *IL4* gene polymorphisms

Genomic DNA was extracted from whole blood using a whole blood DNA extraction kit (Bangalore Genei, Bangalore, India). Polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (PCR-RFLP) analyses were used to genotype -590 C/T and IVS3 of the *IL4* gene, respectively, using the primers shown in Table S2. The restriction enzyme used was *Ava*II (Fermentas, Vilnius, Lithuania) for digesting amplicons of the -590 SNP. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently) and also confirmed by sequencing.

RNA extraction and cDNA synthesis

Total RNA from whole blood was isolated and purified using the Ribopure™ blood Kit (Ambion Inc., Austin, TX, U.S.A.) following the manufacturer's protocol. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas).

Determination of *IL4* and *GAPDH* mRNA expression by real-time polymerase chain reaction

The levels of *IL4* (target) and *GAPDH* (reference) transcripts were measured by real-time PCR using gene-specific primers (Eurofins, Bangalore, India) as shown in Table S2. Real-time PCR was performed in duplicates in 20 μL using LightCycler®480 SYBR Green I Master following the manufacturer's instructions and carried out in the Light Cycler®480 II Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). At the end of the amplification phase a melting curve analysis was carried out on the product formed. The fluorescent data collection was performed during the extension step. The value of Ct was determined by the first cycle number at which fluorescence was greater than the set threshold value. The data are shown as the ratio of Ct values of *IL4* and *GAPDH*.

Estimation of serum interleukin-4 levels by enzyme-linked immunosorbent assay

Serum levels of IL-4 in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA)

using the AviBion Human IL-4 ELISA kit (Ani Biotech Oy, Vantaa, Finland) as per the manufacturer's protocol.

Estimation of serum IgE levels by electrochemiluminescence immunoassay

Serum IgE levels in patients with vitiligo and controls were also monitored by electrochemiluminescence immunoassay (ECLIA) using the Human IgE ECLIA kit (Roche Diagnostics GmbH) as per the manufacturer's protocol.

Statistical analysis

The distribution of the genotypes and allele frequencies was compared using the χ^2 test with 3×2 and 2×2 contingency tables, respectively, using Prism 4 software (GraphPad Software Inc., San Diego, CA, U.S.A.). Vitiligo samples from North India and Gujarat were compared with their respective unaffected controls using χ^2 analysis and Fisher's exact test and strength of associations was estimated by odds ratio (OR) and 95% confidence interval (CI) using the Stata 9.2 statistical program (Statacorp, College Station, TX, U.S.A.). Haplotype analysis was carried out using SHEsis program.[18,19] The linkage disequilibrium (LD) coefficients $D' = D/D_{max}$ and r^2 values for the pair of the most common alleles at each site were also estimated using the Haploview program version 4.1.[20] Differences were considered to be statistically significant if the P-value was $\leq 0{\cdot}05$. Relative gene expression of IL4, serum IL-4 and IgE levels were plotted and analysed by nonparametric unpaired t-test using Prism 4 software. The statistical power of detection of the association with the disease at the 0·05 level of significance was determined by using the G*Power software.[21]

Results

Association of *IL4* gene intron 3 VNTR with vitiligo

The three genotypes of IL4 IVS3 were classified as: R1 (183 bp–two 70 bp repeat allele), R2 (253 bp–three 70 bp repeat allele) and R1R2 (both 183 and 253 bp fragments) (Fig. S1a; see Supporting information).

IVS3 in the IL4 gene was found to be associated with vitiligo susceptibility in the Gujarati population, as genotype and allele frequencies were significantly different ($P < 0{\cdot}0001$, $P < 0{\cdot}0001$, respectively) between the patients and controls. The allele frequencies of the R1 and R2 alleles differed significantly between patients with generalized vitiligo and controls from Gujarat ($P < 0{\cdot}0001$), with R2 being significantly increased in patients compared with controls; however, this was not the case in the North Indian population ($P = 0{\cdot}32$) (Table 1). Genotype R2R2 was significantly increased ($P = 0{\cdot}02$) and R1R1 was significantly reduced ($P < 0{\cdot}0001$) in patients with generalized vitiligo compared with controls (Table 1) in the Gujarat population. Only the R1R1 genotype was significantly reduced in the North Indian patients with generalized vitiligo compared with controls ($P = 0{\cdot}005$).

However, genotype and allele frequencies for IVS3 were not significantly different in patients with localized vitiligo compared with controls in either of the populations (Table 2). This study has 97% and 94% statistical power, respectively, for the Gujarat and North Indian populations for the effect size 0·1 to detect association of IVS3 in patients with vitiligo and the control population ($P < 0{\cdot}05$).

Association of -590 C/T promoter polymorphism in *IL4* gene with vitiligo

The genotyping of the -590 C/T SNP revealed a 195-bp undigested product corresponding to the T allele and 177- and 18-bp digested products corresponding to the C allele (Fig. S1b).

The -590 C/T SNP was found to be associated with vitiligo susceptibility in both the populations, as genotype and allele frequencies were significantly different ($P < 0{\cdot}0001$, $P < 0{\cdot}0001$, respectively) between patients and controls. The frequencies of the C and T alleles differed significantly between patients with generalized vitiligo and controls from Gujarat ($P < 0{\cdot}0001$) with the T allele being significantly increased in patients compared with controls; however, in North Indians, the C allele was significantly increased in patients with generalized vitiligo compared with controls ($P < 0{\cdot}0001$) (Table 1). Genotype TT was significantly increased ($P = 0{\cdot}005$) and CC was significantly reduced ($P = 0{\cdot}0002$) in patients with generalized vitiligo compared with controls (Table 1) in the Gujarat population; however, the CC genotype was significantly increased ($P < 0{\cdot}0001$) and the TT genotype was significantly reduced in the North Indian patients with vitiligo compared with controls ($P < 0{\cdot}0001$).

Interestingly, genotype and allele frequencies for the -590 C/T SNP also differed significantly between patients with localized vitiligo and controls in both the populations ($P = 0{\cdot}004$, $P = 0{\cdot}001$ for Gujarat and $P < 0{\cdot}0001$, $P < 0{\cdot}0001$ for North India) suggesting an association of -590 C/T polymorphism with localized vitiligo (Table 2). This study has 97% and 93% statistical power, respectively, for Gujarat and North Indian populations for the effect size 0·1 to detect an association of -590C/T SNP at $P < 0{\cdot}05$ in patients and the control population.

Linkage disequilibrium and haplotype analyses

As -590C/T and IVS3 are on the same gene it is important to know which of the alleles at the two regions are present together on the same chromosome, i.e. which alleles are in linkage disequilibrium (LD) and make haplotypes. The LD analysis revealed that the two polymorphisms investigated in the IL4 gene were in low LD with $D' = 0{\cdot}072$ and 0·275 in Gujarati and North Indian patients with generalized vitiligo, and $D' = 0{\cdot}159$ and 0·277 in patients with localized vitiligo from Gujarat and North India, respectively.

The estimated frequencies of the haplotypes differed significantly between patients with generalized vitiligo and controls

Table 1 Association study for IL4 gene intron 3 VNTR (IVS3) and -590 C/T single nucleotide polymorphism (SNP) in patients with generalized vitiligo and controls from Gujarat and North India

Population	SNP/genotype	Patients		Controls		P-value	Odds ratio (95% CI)	P-value (global)
		n	Frequency	n	Frequency			
Gujarat	Intron 3 VNTR (IVS3)	395		744				
	Genotype							
	R1R1	23	0·06	106	0·14	0·0001	0·3 (0·2–0·6)	< 0·0001
	R1R2	107	0·27	188	0·25	0·50	1·0 (0·8–1·4)	
	R2R2	265	0·67	450	0·61	0·02	1·3 (1·0–1·7)	
	Allele							
	R1	153	0·19	400	0·27	0·0001	0·6 (0·5–0·8)	< 0·0001
	R2	637	0·81	1088	0·73	0·0001	1·5 (1·2–1·9)	
North India	(IVS3)	464		397				
	Genotype							
	R1R1	13	0·03	27	0·07	0·005	0·3 (0·1–0·8)	0·013
	R1R2	140	0·30	103	0·26	0·16	1·2 (0·9–1·6)	
	R2R2	311	0·67	267	0·67	0·94	0·9 (0·7–1·3)	
	Allele							
	R1	166	0·18	157	0·20	0·31	0·8 (0·6–1·1)	0·323
	R2	762	0·82	637	0·80	0·31	1·1 (0·8–1·4)	
Gujarat	-590 C/T (rs2243250)	395		646				
	Genotype							
	CC	169	0·43	353	0·55	0·0002	0·6 (0·4–0·8)	0·001
	CT	99	0·25	136	0·21	0·13	1·2 (0·9–1·7)	
	TT	127	0·32	157	0·24	0·005	1·4 (1·1–1·9)	
	Allele							
	C	437	0·55	842	0·65	$7·30 \times 10^{6}$	0·6 (0·5–0·7)	< 0·0001
	T	353	0·45	450	0·35	$7·30 \times 10^{6}$	1·5 (1·2–1·8)	
North India	-590 C/T (rs2243250)	464		389				
	Genotype							
	CC	286	0·62	115	0·29	$8·90 \times 10^{21}$	3·8 (2·8–5·1)	< 0·0001
	CT	131	0·28	100	0·26	0·40	1·1 (0·8–1·5)	
	TT	47	0·10	174	0·45	$1·50 \times 10^{30}$	0·13 (0–0·2)	
	Allele							
	C	703	0·75	330	0·42	$9·80 \times 10^{45}$	4·2 (3·4–5·2)	< 0·0001
	T	225	0·25	448	0·58	$9·80 \times 10^{45}$	0·2 (0·1–0·2)	

CI, confidence interval.

in Gujarati and North Indian populations (global $P < 10^{-8}$, $P < 10^{-16}$, respectively) (Table 3). Also, patients with localized vitiligo exhibited significantly different frequencies of haplotypes compared with controls in Gujarat and North Indian populations (global $P < 10^{-5}$, $P < 10^{-30}$, respectively (Table 4).

The results showed that haplotype TR2 was significantly increased ($P < 10^{-9}$) in patients with generalized vitiligo from Gujarat and haplotype CR1 was significantly reduced ($P = 0·001$) compared with controls (Table 3). On the other hand, haplotype CR2 was significantly increased ($P < 10^{-15}$) in patients with generalized vitiligo from North India and TR2 was significantly reduced ($P < 10^{-16}$) compared with the ethnically matched controls. Haplotype analysis for patients with localized vitiligo and controls showed that the TR1 haplotype was increased significantly ($P = 0·031$) in the patients from Gujarat and the CR1 haplotype was reduced significantly compared with controls ($P < 10^{-5}$). The CR2 haplotype was significantly increased ($P < 10^{-15}$) and TR2 was significantly reduced ($P < 10^{-15}$) in patients with localized vitiligo from North India (Table 4). The differences observed in the Gujaratis and North Indians could be due to different ethnicity as they form two independent endogamous groups which do not intermarry.

Age of onset of vitiligo and *IL4* haplotypes in patients with vitiligo

In addition, age of onset of the disease and IL4 haplotypes were also correlated in patients with vitiligo from Gujarat and North India. Interestingly, Gujarati patients with vitiligo with the TTR2R2 haplotype had an early onset of vitiligo (mean age ± SD, 9·5 ± 0·8660 years) compared with the CCR1R2 and TTR1R1 haplotypes ($P = 0·027$; $P = 0·005$, respectively) (Fig. 1a). Also, patients with the TTR1R2 haplotype had early onset of vitiligo (mean age ± SD, 11·29 ± 1·742 years) com-

Table 2 Association study for IL4 gene intron 3 VNTR (IVS3) and -590 C/T single nucleotide polymorphism (SNP) in patients with localized vitiligo and controls from Gujarat and North India

Population	SNP/genotype	Patients		Controls		P-value	Odds ratio (95% CI)	P-value (global)
		n	Frequency	*n*	Frequency			
Gujarat	Intron 3 VNTR (IVS3)	110		744				
	Genotype							
	R1R1	15	0·13	106	0·15	0·80	0·9 (0·4–1·7)	0·807
	R1R2	25	0·23	188	0·25	0·50	0·8 (0·5–1·4)	
	R2R2	70	0·64	450	0·60	0·50	1·1 (0·7–1·7)	
	Allele							
	R1	55	0·25	400	0·27	0·61	0·9 (0·6–1·2)	0·624
	R2	165	0·75	1088	0·73	0·61	1·1 (0·7–1·5)	
North India	IVS3	132		397				
	Genotype							
	R1R1	7	0·05	27	0·07	0·50	0·7 (0·2–1·8)	0·734
	R1R2	38	0·29	103	0·26	0·50	1·1 (0·7–1·8)	
	R2R2	87	0·66	267	0·67	0·70	0·9 (0·6–1·4)	
	Allele							
	R1	52	0·20	157	0·20	0·90	0·9 (0·6–1·4)	0·930
	R2	212	0·80	637	0·80	0·90	1·0 (0·7–1·4)	
Gujarat	-590 C/T	110		646				
	Genotype							
	CC	43	0·39	353	0·55	0·002	0·5 (0·3–0·8)	0·004
	CT	37	0·34	136	0·21	0·003	1·9 (1·1–3·0)	
	TT	30	0·27	157	0·24	0·500	1·1 (0·7–1·8)	
	Allele							
	C	123	0·56	842	0·65	0·008	0·6 (0·5–0·9)	0·001
	T	97	0·44	450	0·35	0·008	1·4 (1·0–1·9)	
North India	-590 C/T (rs2243250)	132		389				
	Genotype							
	CC	82	0·62	115	0·29	$2·60 \times 10^{11}$	3·9 (2·5–6·0)	< 0·0001
	CT	34	0·25	100	0·26	0·9	1·1 (0·6–1·6)	
	TT	16	0·13	174	0·45	$1·70 \times 10^{11}$	0·17 (0·09–0·3)	
	Allele							
	C	198	0·75	330	0·42	$5·60 \times 10^{20}$	4 (2·9–5·6)	< 0·0001
	T	66	0·25	448	0·58	$5·60 \times 10^{20}$	0·24 (0·1–0·3)	

CI, confidence interval.

Table 3 Haplotypes of IL4 -590 C/T and intron 3 VNTR showing significant differences between patients with generalized vitiligo and controls from Gujarat and North India

Population	Haplotype (-590 C/T and intron 3 VNTR)	Patients Frequency (%)	Controls Frequency (%)	P-value	P-value (global)	Odds ratio (95% CI)
Gujarat		2n = 598	2n = 1246			
	CR1	9·00	14·33	0·001	$< 10^{-8}$	0·590 (0·428–0·815)
	CR2	49·70	5·25	0·026		0·801 (0·658–0·973)
	TR1	7·10	8·70	0·229		0·798 (0·551–1·154)
	TR2	34·20	21·72	$< 10^{-9}$		1·877 (1·512–2·329)
North India		2n = 898	2n = 650			
	CR1	7·69	7·55	0·914	$< 10^{-16}$	1·021 (0·698–1·494)
	CR2	68·59	33·68	$< 10^{-15}$		4·298 (3·466–5·331)
	TR1	9·79	11·84	0·197		0·808 (0·584–1·118)
	TR2	13·93	46·93	$< 10^{-16}$		0·183 (0·143–0·234)

CI, confidence interval.

Table 4 Haplotypes of IL4 -590 C/T and intron 3 VNTR showing significant differences between patients with localized vitiligo and controls from Gujarat and North India

Population	Haplotype (-590 C/T and intron 3 VNTR)	Patients Frequency (%)	Controls Frequency (%)	P-value	P-value (global)	Odds ratio (95% CI)
Gujarat		2n = 184	2n = 1246			
	CR1	2·67	14·33	$< 10^{-5}$	$< 1\ 0^{-5}$	0·164 (0·066–0·408)
	CR2	60·37	55·25	0·191		1·234 (0·900–1·692)
	TR1	13·63	8·70	0·031		1·657 (1·041–2·637)
	TR2	23·33	21·72	0·623		1·096 (0·759–1·583)
North India		2n = 264	2n = 650			
	CR1	4·12	7·55	0·058	$< 10^{-30}$	1·021 (0·698–1·494)
	CR2	71·64	33·68	$< 10^{-15}$		4·298 (3·466–5·331)
	TR1	14·82	11·84	0·220		0·808 (0·584–1·118)
	TR2	9·42	46·93	$< 1\ 0^{-15}$		0·183 (0·143–0·234)

CI, confidence interval.

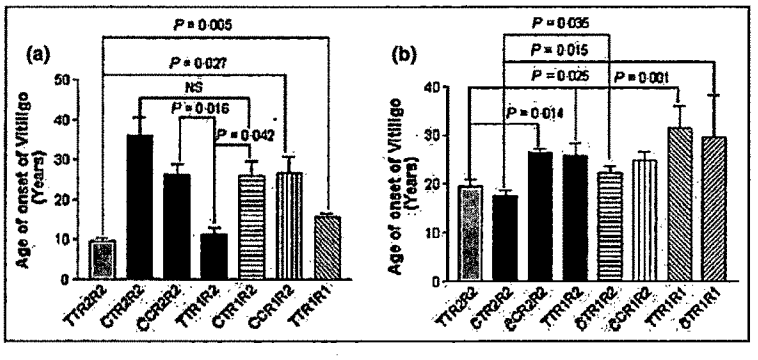

Fig 1. Comparison of age of onset of vitiligo with IL4 haplotypes in patients with vitiligo from (a) Gujarat and (b) North India.

pared with those with CCR2R2 and CTR1R2 (P = 0·016; P = 0·042, respectively) (Fig. 1a).

Interestingly, North Indian patients with vitiligo with the TTR2R2 haplotype also had an early onset of the disease (mean age ± SD, 19·56 ± 1·283 years) compared with those with CCR2R2, TTR1R2 and TTR1R1 (P = 0·014; P = 0·025; P = 0·0013, respectively). Moreover, patients with the CTR2R2 haplotype also had early onset of vitiligo (mean age ± SD, 17·54 ± 1·194 years) compared those with CTR1R1 and CTR1R2 (P = 0·015; P = 0·035, respectively) (Fig. 1b).

Relative gene expression of *IL4* in patients with generalized vitiligo and controls from Gujarat

Gene expression studies revealed higher expression of IL4 in 84 patients with generalized vitiligo compared with 93 controls (P = 0·001) (Fig. 2a). Further, the expression levels of IL4 were analysed with respect to haplotypes generated from the two investigated polymorphisms of IL4 (Fig. 2b). IL4 mRNA expression differed significantly with respect to haplotypes generated at -590 and IVS3 loci, i.e. CCR2R2, CTR2R2

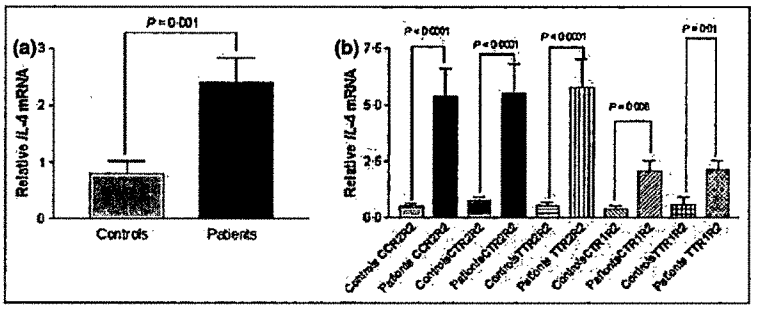

Fig 2. Relative gene expression and genotype–phenotype correlation for IL4 transcript in controls and patients with generalized vitiligo. (a) Relative gene expression of IL4 in 84 patients with generalized vitiligo and 93 controls as suggested by ratio of target (IL4 transcripts)/reference (GAPDH transcripts). (b) Relative mRNA expression of IL4 with respect to -590 C/T and intron 3 VNTR haplotypes in 84 patients with generalized vitiligo and 93 controls as suggested by ratio of target (IL4 transcripts)/reference (GAPDH transcripts).

and TTR2R2 ($P < 0{\cdot}0001$; $P < 0{\cdot}0001$; $P < 0{\cdot}0001$, respectively) in patients with vitiligo compared with controls. Also, the CTR1R2 and TTR1R2 genotypes revealed significantly higher mRNA expression ($P = 0{\cdot}006$; $P = 0{\cdot}01$, respectively) in patients compared with controls.

Estimation of serum interleukin-4 levels and its correlation with investigated polymorphisms in patients with generalized vitiligo and controls from Gujarat

Serum IL-4 levels in 86 patients with generalized vitiligo and 95 controls were estimated by ELISA. IL-4 levels were found to be significantly different in patients with vitiligo compared with controls ($P < 0{\cdot}0001$) (Fig. 3a). In addition, serum IL-4 levels were analysed with respect to haplotypes generated at the -590 and IVS3 loci. IL-4 levels were significantly higher in patients for the CCR2R2, CTR2R2, CTR1R2 and TTR2R2 genotypes ($P = 0{\cdot}002$; $P = 0{\cdot}013$; $P = 0{\cdot}044$ and $P = 0{\cdot}002$, respectively) (Fig. 3b).

Estimation of serum IgE levels and its correlation with investigated polymorphisms in patients with generalized vitiligo and controls from Gujarat

We also measured serum IgE levels in 82 patients with generalized vitiligo and 90 controls from Gujarat. Patients with vitiligo showed increased IgE levels compared with controls ($P = 0{\cdot}019$) (Fig. 4a). Moreover, when serum IL-4 levels were compared with respect to haplotypes generated at -590 and IVS3 loci, the IgE levels were significantly increased for TTR2R2, CTR2R2, CCR2R2, TTR1R2 and CTR1R2 haplotypes ($P = 0{\cdot}003$, $P = 0{\cdot}014$, $P = 0{\cdot}033$, $P = 0{\cdot}006$ and $P = 0{\cdot}026$, respectively) in patients compared with controls (Fig. 4b). However, no significant difference was observed for serum IgE levels between patients and controls for CCR1R2 and TTR1R1 haplotypes ($P = 0{\cdot}579$, $P = 0{\cdot}587$, respectively) (Fig. 4b).

Discussion

Although the aetiology of vitiligo remains obscure, autoimmunity has been suggested to play a major role in its pathogenesis.[5] Our previous study suggested that 22% of patients with vitiligo from Gujarat exhibit a positive family history and 14% patients have at least one-first-degree relative affected.[22] The destruction of melanocytes due to an autoimmune response in vitiligo can be either through cellular and/or humoral immune response.[4,5] We have also shown that 66% of patients with vitiligo possess antimelanocyte antibodies in their circulation compared with a control population.[23] Recently, we showed a positive association of *HLA-A**33:01, *HLA-B**44:03 and *HLA-DRB1**07:01 with vitiligo in patients from North India and Gujarat suggesting an autoimmune link of vitiligo in these cohorts.[24] The genotype–phenotype correlation of *CTLA4* gene

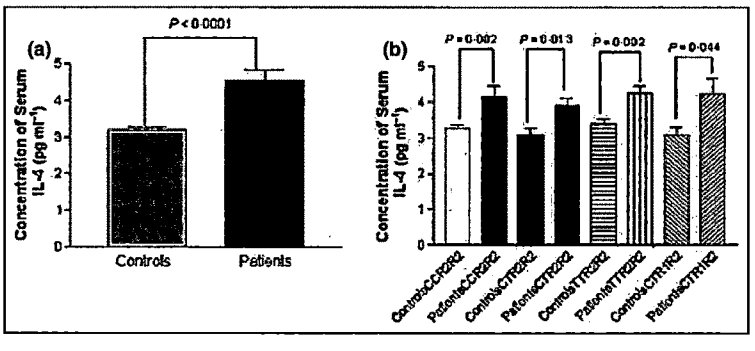

Fig 3. Correlation of serum interleukin (IL)-4 levels with investigated polymorphisms in controls and patients with generalized vitiligo. (a) Comparison of serum IL-4 levels (pg mL^{-1}) in 86 patients with generalized vitiligo and 95 controls as determined by enzyme-linked immunosorbent assay. (b) Comparison of serum IL-4 levels (pg mL^{-1}) in 86 patients with generalized vitiligo and 95 controls with respect to haplotypes based on the investigated polymorphisms.

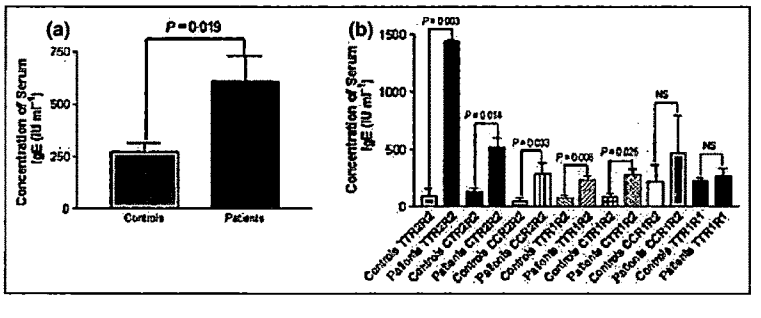

Fig 4. Correlation of serum IgE levels with investigated polymorphisms in controls and patients with generalized vitiligo. (a) Comparison of serum IgE levels (IU mL^{-1}) in 82 patients with generalized vitiligo and 90 controls as determined by electrochemiluminescence immunoassay. (b) Comparison of serum IgE levels (IU mL^{-1}) in 82 patients with generalized vitiligo and 90 controls with respect to haplotypes based on the investigated polymorphisms.

polymorphisms also supports the autoimmune pathogenesis of vitiligo in the Gujarat population whereas our earlier studies on MBL2, ACE and PTPN22 polymorphisms did not show significant association.[25–28]

In this study, we investigated two well-documented IL4 polymorphisms, -590 C/T SNP and IVS3, in patients with vitiligo from Gujarat and North India. The -590 T allele is associated with increased promoter activity, a higher proportion of IL-4-producing Th cells,[17] and elevated serum IgE level.[15] One study by Pehlivan *et al.*[29] in 2009 reported its nonassociation with vitiligo in a Turkish population. Interestingly, ours is the first report suggesting a strong association of -590 C/T polymorphism with vitiligo susceptibility. We found that the susceptible (TT) genotype frequency is higher in patients from Gujarat with generalized and with localized vitiligo compared with controls, suggesting the profound effect of -590 C/T polymorphism in both the types of vitiligo. This particular genotype confers higher expression of IL4, as the T allele is known to increase transcriptional activity[15] and thereby also upregulates IL-4 protein expression. However, the CC genotype frequency was higher in patients with vitiligo from North India, suggesting a difference in genetic predisposition due to ethnicity differences between the two populations. Also, higher frequency of the low producing genotype CC in North Indian patients may implicate the role of Th1 type of autoimmune responses in these patients compared with higher Th2 responses in Gujarat. It is pertinent to mention here that the prevalence of vitiligo in Gujarat is higher than in North India and it is possible that different autoimmune aetiologies may be playing a role in the manifestation of the disease in the two ethnic groups in India. The IL4 -590 C/T promoter polymorphism is associated with autoimmune disorders such as allergic asthma and systemic lupus erythematosus (SLE).[30,31] The IL4 -590 TT genotype was significantly more frequent in patients with rheumatoid arthritis (RA) than in controls.[32] Nevertheless there are contradictory reports which suggest that this polymorphism is not associated with autoimmune disorders such as autoimmune thyroid disease[33] and Graves disease.[34]

The present study also addressed IVS3 in patients with vitiligo and controls and found a significant association with vitiligo susceptibility in the Gujarati population. It is suggested that a distinct number of VNTR copies might affect the transcriptional activity of the IL4 gene.[35] The three repeat allele (R2) is known to be high producer of IL-4,[17] which was found to be higher in patients with generalized vitiligo from Gujarat; however the allele has no association with patients with localized vitiligo from Gujarat suggesting that it may have a crucial role in generalized vitiligo. Previously, this polymorphism was reported to be associated with RA and immune thrombocytopenic purpura and SLE.[35,36] One North Indian study also suggested an association of IVS3 with susceptibility to type-2 diabetes.[37] In contrast, this polymorphism was not found to be associated with autoimmune thyroid disease.[33] This is the first report investigating the role of IVS3 and suggesting its positive association with vitiligo.

Our relative gene expression studies and ELISA results showed a significant increase in IL4 transcript and protein levels in Gujarati patients with vitiligo suggesting its crucial role in vitiligo pathogenesis. This is the first report where IL4 expression and protein levels were studied in patients with vitiligo. Further, considering the IL4 -590 C/T promoter polymorphism, we found that patients with the CT and TT genotypes revealed higher IL4 mRNA expression. Also, the CT and TT genotypes showed significant differences in serum IL-4 levels in Gujarati patients with vitiligo compared with controls. In a recent study by Kim *et al.*,[38] the -590 C/T SNP was shown to be associated with higher IL4 mRNA expression in patients with asthma. Our results document the same evidence in patients with vitiligo. We found that patients with vitiligo harbouring the CCR2R2, CTR2R2 and TTR2R2 haplotypes showed significantly increased mRNA and protein levels compared with controls, revealing the profound effect of the R2R2 genotype on IL-4 expression.

In addition, higher serum IgE levels in Gujarati patients with vitiligo suggest that this increase could be due to increased IL-4 levels. Further, the -590 TT and IVS3 R2R2 genotypes were found to be associated with increased serum IgE levels. Our results are in accordance with those of Guia and Ramos, demonstrating the association of -590 TT genotype with elevated serum IgE levels in individuals with atopic allergy.[39] Our results showed that patients with vitiligo with the CCR2R2, CTR2R2 and TTR2R2 haplotypes showed significantly increased serum IgE levels compared with controls, revealing the intense effect of the R2R2 genotype.

Previous studies also showed an increase in total IgE count in 59 patients with vitiligo and these patients had a significantly higher incidence of vitiligo in their families, an earlier onset and a rapid worsening of the disease.[40,41] In particular, analysis of age of onset of the disease in patients suggests that TTR2R2, TTR1R2 and CTR2R2 had a significant effect in the early onset of the disease and further can also be supported by the increased serum IL-4 and IgE levels in patients with these haplotypes. Thus, our findings strongly support the fact that the T and R2 alleles are associated with increased promoter activity,[15] a higher proportion of IL-4-producing Th cells[17] and elevated serum IgE levels.[15] Considering IL4 IVS3, the R2 allele is known to be a higher producer of IL-4.[17]

In summary, our present study on the well-documented IL4 gene polymorphisms reveals the crucial role of IL-4 in vitiligo susceptibility of the Gujarati population; however, only the -590 C/T polymorphism showed significant association with vitiligo in the North Indian population. The differences in the results in the two populations studied seem very interesting and suggest different pathways may be involved in achieving the same goal, i.e. vitiligo. It is well known that IL-4 has been shown to stimulate B-cell proliferation, to regulate immunoglobulin class switching (IgG1 and IgE), and to promote T-cell development,[10] and therefore plays an important role in precipitating autoimmune responses. Considering the fact that IL-4 is one of the most important Th2 cytokines, and increased IL-4 levels induce a balance shift from Th1 to Th2

cells, determining the molecular profile of a particular person becomes crucial in understanding the immune reaction mechanisms. In cases of atopic dermatitis where hyper-produced Th1 and Th2 cytokines are involved, the initiation phase of the disease involved increased IL-4 production by Th2 cells.[42] Previously, an *in vivo* study showed that IL-4 contributed to increased levels of IgE and IgG1 in mice treated with mercuric chloride, which led to a systemic autoimmune disease, emphasizing the role of IL-4 in humoral immune response-mediated pathogenesis.[43]

We therefore hypothesize that IL-4 plays a crucial role in aggravating immune responses in Gujarati patients with vitiligo, and its ability to stimulate B-cell proliferation may be involved in precipitating the humoral immune responses (Th2) in these patients. Also, for the first time, we document the role of the IL4 -590 C/T promoter polymorphism as well as IVS3 in increased expression of IL4 transcript in correlation to higher IL-4 and serum IgE levels in patients with vitiligo and thereby conferring susceptibility towards vitiligo in the Gujarati population.

What's already known about this topic?

- Interleukin (IL)-4 has been shown to stimulate B-cell proliferation, to regulate immunoglobulin class switching (IgG1 and IgE) and to promote T-cell development, and therefore may play an important role in autoimmunity.

What does this study add?

- The present study suggests that intron 3 VNTR and -590 C/T single nucleotide polymorphism of IL4 may be genetic risk factors for vitiligo susceptibility in the Gujarati population and be responsible for the altered levels of IL-4 and IgE in patients with vitiligo, thereby indicating the crucial role of IL-4 in autoimmune pathogenesis of vitiligo.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis of IL4 -590 C/T polymorphism on 10% polyacrylamide gel electrophoresis. Lanes 1, 3 and 5, heterozygous (CT) genotypes; lane 2, homozygous (CC) genotype; lane 4, homozygous (TT) genotype; lane M, 100 bp DNA ladder. (b) PCR analysis of IL4 intron 3 VNTR polymorphism on 2% agarose gel electrophoresis. Lanes 1, 5 and 8, homozygous (R2R2) genotypes; lanes 2 and 6, heterozygous (R1R2) genotypes; lanes 3, 4 and 7, homozygous (R1R1) genotypes; lane M, 50 bp DNA ladder.

Table S1. Demographic characteristics of patients with vitiligo and unaffected controls from Gujarat and North India.

Table S2. Primers used for genotyping of IL4 intron 3 VNTR (IVS3) and -590 C/T single nucleotide polymorphism and IL4 gene expression analysis.

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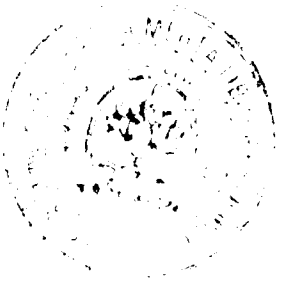

SYNOPSIS

INTRODUCTION

Genes involved in common complex diseases such as autoimmune disorders that affect approximately 5% of the population remain obscure. Vitiligo is an acquired hypomelanotic disorder characterized by circumscribed depigmented macules in the skin resulting from loss of functional melanocytes from the epidermis. This is a cosmetic disfigurement disorder and may lead to psychological and social problems particularly in brown and black people. It affects 0.5-1% of the world population (Taieb et al., 2007) and in India the incidence of vitiligo is found to be 0.5-2.5% (Handa and Kaur, 1999). The etiology of vitiligo is still unknown, but it is hypothesized to be of autoimmune origin due to its frequent association with several autoimmune diseases, the presence of circulating autoantibodies and autoreactive T-cells in the sera of vitiligo patients compared to unaffected individuals (Kemp et al., 2001).

Various factors such as oxidative stress, genetic factors, neural factors, toxic metabolites and lack of melanocyte growth factors might contribute for precipitating the disease in susceptible people (Njoo and Westerhof, 2001). The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Nordlund, 1997). It has been found that about 20% of vitiligo patients have at least one first-degree relative affected (Nath et al., 1994). Our study also showed that 22% of Gujarat vitiliginous patients exhibit positive family history and 14% patients have at least one first-degree relative affected (Shajil et al., 2006). Vitiligo does not follow the simple Mendelian inheritance pattern and its mode of heredity suggests that it is a polygenic disease.

Furthermore, several genes that play role in regulating immunity have been found to be associated with susceptibility to vitiligo including allelic variants in the cytotoxic T -lymphocyte antigen-4 gene (*CTLA4*) (Blomhoff et al., 2005), the autoimmune susceptibility loci (*AIS*1, *AIS*2, *AIS*3 and *SLEV*1) (Alkhateeb et al., 2001; Fain et al., 2003; Spritz et al., 2004), the autoimmune regulator (*AIRE*) gene (Nagamine et al., 1997), NACHT leucine-rich-repeat protein 1 (*NALP*1- maps at *SLEV*1 sucsceptibility locus) (Jin et al., 2007), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) (Laberge et al., 2008) and certain human leukocyte antigen specificities of the major histocompatibility complex (Le Poole et al., 2008; Fain et al., 2006).

Most of the population studies on vitiligo are done in western countries; however clinical studies on Indian population are scarce. Since the environmental factors could account for the origin of autoimmunity in the susceptible patients, clinical and genetic studies on vitiligo in Indian patients are required. In order to explore the genetic susceptibility, identification and systematic study of genes involved in regulation of autoimmunity is essential. Attempts to identify genes involved in vitiligo susceptibility have involved gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses. The aim of present study was to assess role of autoimmunity in pathogenesis of vitiligo through candidate gene approach.

Objectives of the present study:

1. Estimation of antimelanocyte antibody levels in Gujarat vitiligo patients compared to controls.
2. Genetic association of Protein Tyrosine Phosphatase Non receptor 22 *(PTPN22)* and Angiotensin Converting Enzyme *(ACE)* genes polymorphisms with vitiligo susceptibility.
3. Role of Mannan Binding Lectin-2 (*MBL2*) gene structural and promoter polymorphisms in vitiligo susceptibility.
4. Role of Interferon-γ (*IFN-γ*) gene intron 1 polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility.
5. Role of Cytotoxic T-Lymphocyte Associated antigen-4 (*CTLA-4*) gene polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility.
6. Role of Melanocyte Proliferating Gene 1 (*MYG1*) promoter and structural polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility.
7. Role of NACHT Leucine-rich Repeat Protein 1 (*NALP1*) gene promoter and structural polymorphisms in vitiligo pathogenesis.
8. Determination of $CD4^+/CD8^+$ ratio and estimation of $CD4^+CD25^+$FoxP3 T-regulatory cells (Tregs) in vitiligo patients and controls.

1. Estimation of antimelanocyte antibody levels in Gujarat vitiligo patients compared to controls:

We have analyzed plasma samples of 300 vitiligo patients and 400 controls for the presence of antimelanocyte antibodies by ELISA. A significant increase in the antimelanocyte antibody levels was seen in vitiligo patients compared to controls ($p=0.001$). Seventy five percent of vitiligo patients had antimelanocyte antibodies in their circulation suggesting that autoimmunity may play an important role in vitiligo pathogenesis.

2. Genetic association of Protein Tyrosine Phosphatase Non receptor 22 *(PTPN22)* and Angiotensin Converting Enzyme *(ACE)* genes polymorphisms with vitiligo susceptibility:

A) Genotyping of PTPN22 1858C/T (rs2476601) polymorphism in vitiligo patients and controls of Gujarat:

Protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene is known to play an important role in negative T cell regulation. *PTPN22* C/T single nucleotide polymorphism at 1858 (rs2476601) position was reported to be associated with increased risk of autoimmune diseases including generalized vitiligo (Canton et al., 2005). A total of 126 vitiligo patients and 140 age matched healthy controls were analyzed for the 1858 C/T polymorphism. The genotype and allele frequencies of this C/T SNP did not differ significantly between control and patient populations ($p= 0.198$; $p=0.560$ respectively) suggesting that there is no association of the C/T (*XcmI*) *PTPN22* marker with vitiligo.

The study could not attain statistical significance consequent to lower prevalence rate of 1858T allele in the studied population. Interestingly, 1858T allele has been found to be absent in East Asians including Japanese (Ban et al., 2005) Chinese (Begovich et al., 2004) and South Asian Indians (Ray et al., 2006). Thus, our results suggest that the 1858T allele may not be a predisposing factor in Gujarat population that contributes in development of vitiligo.

B) Genotyping of ACE I/D polymorphism (NCBI: AF118569) in vitiligo patients and controls of Gujarat:

Angiotensin converting enzyme (*ACE*) has been found to be associated with several autoimmune disorders (Scholzen et al., 2003). An insertion/deletion (I/D) polymorphism of a 287-base pair repetitive sequence in intron 16 of the *ACE* gene (NCBI: AF118569; repeat region 14094–14381) is reported to be associated with the development of vitiligo (Jin et al., 2004). The I/D polymorphism of the *ACE* gene accounts for the variability of serum ACE activity, D/D genotype having the highest and I/I genotype having the lowest ACE activity (Rigat et al., 1990).

We investigated the distribution of *ACE* I/D genotypes in 125 vitiligo patients and 156 ethnically matched healthy controls of Gujarat population to find the relationship between *ACE* I/D polymorphism and vitiligo. No significant difference in the genotype frequencies of I/I, I/D and D/D genotypes was observed between vitiligo patients and control subjects (p=0.459), suggesting that there is no association of the *ACE* I/D polymorphism with vitiligo. The allele frequencies of *ACE* I/D polymorphism did not differ significantly between the controls and patient population (p=0.252).

Previously, the D allele of the *ACE* gene was reported to confer susceptibility to vitiligo in Korean population (Jin et al., 2004). However, the current study suggests that the polymorphic variant D allele is not associated with vitiligo susceptibility. This may be because of the differences in ethnicity and/or geographic location of the subjects under study. Our results are comparable to the previous report in which genotype frequencies for the I/D polymorphism were not significantly different between controls and generalized vitiligo patient population (Akhtar et al., 2005) suggesting that the well documented I/D polymorphism in intron 16 of angiotensin converting enzyme in Gujarat population may not be associated with vitiligo susceptibility.

3. Genetic association of Mannan binding lectin-2 (*MBL2*) gene structural and promoter polymorphisms with vitiligo susceptibility:

Mannan-binding lectin (MBL) [mannose binding lectin (MBL)] is a liver-derived calcium dependent serum protein, which plays an important role in innate immune defense. Possible role of MBL in vitiligo pathogenesis could be due to defective clearance of apoptotic cells which may substantially contribute to the triggering of

autoimmune responses (Werth et al., 2002). The functional *MBL2* gene is located on chromosome 10 (q11.2-q21) and comprises of four exons. Three functional single-nucleotide polymorphisms (SNPs) in exon 1 of *MBL2* gene have been reported: codon 54 (GGC→GAC; designated B allele), codon 57 (GGA→GAA; designated C allele), and codon 52 (CGT→TGT; designated D allele) (Lipscombe et al., 1992). These variant alleles in the collagen like domain cause structural changes in MBL and will lead to its low plasma levels and thus results in plasma concentrations varying by more than 1000-fold within the population (5 ng/ml to 5 μg/ml) (Garred et al., 2003).
We investigated the association of *MBL2*-deficient genotypes of both promoter and exon 1 with vitiligo susceptibility in Gujarat population. A total of 92 vitiligo patients and 94 unaffected age matched controls of Gujarat population participated in this study, out of which 62 patients and 72 age matched controls were genotyped for codon 52 (allele D), 84 patients and 81 controls were genotyped for codon 54 (allele B), and 68 patients and 72 controls were genotyped for codon 57 (allele C). For genotyping of -221 promoter polymorphism, 92 patients and 93 control subjects were used. The genotyping of *MBL2* structural and promoter polymorphisms were carried out by heteroduplex analysis.

A) Genotyping of MBL2 codon 52 (rs5030737), codon 54 (rs1800450) and codon 57 (rs1800451) polymorphisms in vitiligo patients and controls of Gujarat:

Codon 52, codon 54 and codon 57 polymorphisms of MBL2 exon 1 were not found to be associated with vitiligo patients (p=0.019 for codon 52, p=0.373 for codon 54, p=0.855 for codon 57). Also, there was no significant difference in allele frequencies of codon 52, codon 54 and codon 57 between patients and controls (p=0.020 for D allele, p=0.378 for B allele and p=0.858 for C allele).
The association study for exon 1 polymorphisms was also done by A/O genotyping where all the three codon polymorphisms were placed in one group designated as 'O' allele and wild type as 'A' allele. There was no significant association found between the A/O genotype and vitiligo (p=0.999). Furthermore, 'O' allele was also not significantly associated with any of the population (p=0.968).

B) Genotyping of MBL2 -221 promoter polymorphism (rs7096206) in vitiligo patients and controls of Gujarat:

The promoter region of the *MBL2* gene contains a number of regulatory elements, which affect transcription of the gene and hence polymorphisms in the promoter region, thus affecting the plasma MBL concentration directly. There was no significant association found between -221 promoter polymorphism and the risk of vitiligo (p=0.889). Also, the allele frequencies of patients and controls for -221 (X/Y) promoter polymorphism did not show significant difference (p=0.765).

Haplotype analysis:

The promoter and structural genotypes of patients and controls were combined to construct haplotypes and to find whether there was any haplotype associated with vitiligo susceptibility. Interestingly, no haplotype was found to be associated with vitiligo when compared with chi-square test (p=0.962). Moreover, the haplotypes were distributed to represent MBL levels as: high (YA/YA, YA/XA), medium (XA/XA, YA/YO) and low (XA/YO, YO/YO) in both controls and patients. However, no significant difference was found in the distribution of high, medium and low haplotypes (p=0.838).

Previously, an association of codon 54 (allele B) with vitiligo was observed in Turkish population where only two codons 54 and 57 were genotyped with smaller sample size (Onay et al., 2007); however, we found there was no association between vitiligo and *MBL2* structural and promoter polymorphisms. This is the first report that shows non-association of structural and promoter polymorphisms of *MBL2* gene with vitiligo. In conclusion, it can be considered that the structural and promoter polymorphisms in the *MBL2* gene may not confer a role in vitiligo susceptibility of Gujarat population.

4. Role of Interferon-γ (*IFN-γ*) gene intron 1 polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility:

Interferon-gamma (IFN-γ) is a key regulator of development and functions of the immune system. Genetic variability in intronic region of *IFN-γ* has been reported to be associated with its enhanced transcription thereby increasing the risk for several autoimmune diseases.

A) Genetic association of IFN-γ +874T/A (rs2430561) polymorphism with vitiligo patients and controls of Gujarat:

A single nucleotide T/A polymorphism at the 5' end of the CA repeat region in the first intron of the *IFN-γ* gene (+874 A/T polymorphism; rs2430561) has been correlated with the presence or absence of the microsatellite allele 2. Also, the presence of *IFN-γ* (+874 T^{hi}/A^{lo}) polymorphism creates a putative NF-κB binding site in intron1 and shows preferential binding to the T allele and correlates with high *IFN-γ* producer phenotype (Pravica et al., 2000). We genotyped *IFN-γ* noncoding +874T/A polymorphism (rs2430561) in 517 vitiligo patients and 881 ethnically, age-matched unaffected individuals by ARMS-PCR. Genotypes were also confirmed by sequencing of few random amplicons.

Intron1 +874T/A polymorphism of *IFN-γ* gene was not found to be associated with vitiligo patients as genotype frequencies did not differ between patients and controls (p=0.485). Also, there was no significant difference in allele frequencies of this polymorphism between patients and controls (p=0.274). Our results are in concordance with those of Namian et al. (2009) suggesting that +874T/A intron1 polymorphism of *IFN-γ* gene has no significant influence on vitiligo susceptibility. Therefore, we propose that *IFN-γ* +874T/A intron1 polymorphism is not a genetic risk factor for vitiligo susceptibility in Gujarat population.

B) Genetic association of IFN-γ CA microsatellite (rs3138557) polymorphism with vitiligo patients and controls of Gujarat:

The CA repeat microsatellite sequence in the first intron is polymorphic. Allele 2, with 12 CA repeats is associated with constitutively high IFN-γ production (*in vitro)* (Pravica et al., 2000). The genotyping of *IFN-γ* CA microsatellite is also being done in vitiligo patients and controls by high resolution melt curve (HRM) analysis using real time PCR and the results will be shown in the thesis.

C) Estimation of IFN-γ protein levels in patients and controls using ELISA:

The levels of IFN-γ in plasma samples of vitiigo patients and controls will be estimated using sandwich ELISA and the results will be shown in the thesis.

D) Establishment of genotype-phenotype correlation with vitiligo susceptibility:

To assess the effect of *IFN-γ* +874 T/A SNP and CA microsatellite, genotypes will be compared for the IFN-γ protein levels between patients and controls and thus genotype-phenotype correlation will be established for vitiligo susceptibility.

5. Role of Cytotoxic T-lymphocyte associated antigen-4 (*CTLA*-4) gene polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility:

CTLA-4 is a negative regulator of T-cell function, which is suggested to be involved in susceptibility to several autoimmune diseases including vitiligo. Genetic variability in *CTLA*-4 gene is reported to be associated with its altered levels thereby increasing the risk for several autoimmune diseases. We explored exon 1 +49A/G (rs231775) and 3' UTR CT60A/G (rs3087243) SNPs in *CTLA*-4 gene and correlated them with *CTLA*-4 transcript levels in vitiligo patients and controls of Gujarat.

A) Analysis of association between CTLA-4 exon 1 +49A/G (rs231775) polymorphism and susceptibility to vitiligo:

The single nucleotide polymorphism: A to G transition at position 49 (A49G) of exon 1 leads to an alanine to threonine amino acid substitution at codon 17 of the leader peptide (A17T) (Nistico et al., 1996). Interestingly, the G allele of *CTLA*-4 +49A/G SNP was reported to be involved in the altered intracellular transport of the CTLA-4 protein and its availability on the cell surface. We analyzed 347 vitiligo patients and 746 controls of Gujarat for *CTLA*-4 +49A/G polymorphism by PCR-RFLP. The genotype and allele frequencies for *CTLA*-4 +49A/G polymorphism did not differ significantly between vitiligo patients and controls (p=0.771; p=0.461 respectively) suggesting the non-association of +49A/G SNP with vitiligo susceptibility. Our results are in line with those of South Indian and Romanian populations (Deeba et al., 2010; Birlea et al., 2009).

B) Analysis of association between CTLA-4 3'UTR CT60A/G (rs3087243) polymorphism and susceptibility to vitiligo:

The 3'UTR CT60A/G allelic variation was reported to be correlated with lower mRNA levels of the soluble alternative splice form of CTLA-4 (sCTLA-4), suggesting its crucial role in autoimmune diseases (Ueda et al., 2003). We genotyped 437 vitiligo patients and 738 controls of Gujarat for *CTLA*-4 CT60A/G polymorphism by PCR-RFLP. The *CTLA*-4 CT60A/G polymorphism was found to be in significant

association with vitiligo patients, since the genotype and allele frequencies differed significantly between patients and controls ($p<0.0001$; $p<0.0001$ respectively). The frequency of CT60GG genotype was significantly higher in vitiligo patients as compared to controls suggesting that 3'UTR CT60A/G may be a genetic risk factor for vitiligo susceptibility in Gujarat population.

Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the two polymorphisms investigated in the *CTLA*-4 gene were in moderate LD association (+49A/G: CT60A/G; D' 0.64, $r^2=0.11$). A haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes did not differ between vitiligo patients and controls (global p-value = 0.123). However, the GG haplotype was more frequently observed in vitiligo patients and increased the risk of vitiligo by 1.2-fold [p=0.048; odds ratio (OR): 1.243; 95% confidence interval (CI): (1.001-1.543)].

C) Relative gene expression of flCTLA-4 and sCTLA-4 in patients and controls:

Analysis of fl*CTLA*-4 and s*CTLA*-4 mRNA expression showed significantly decreased expression in patients after normalization with *GAPDH* expression (p=0.007 and p=0.037 respectively). The $2^{-\Delta\Delta Cp}$ analysis showed approximately two fold change in the expression of fl*CTLA*-4 and s*CTLA*-4 mRNA expression in patients as compared to controls.

D) Establishment of genotype-phenotype correlation with vitiligo susceptibility:

We analyzed the mRNA expression of fl*CTLA*-4, s*CTLA*-4 based on the +49A/G and CT60A/G genotypes of 76 vitiligo patients and 83controls. The expression levels of fl*CTLA*-4 for AA, AG and GG genotypes of exon 1 +49A/G polymorphism did not differ significantly in vitiligo patients as compared to controls (p=0.122, p=0.320 and p=0.068 respectively). Also, s*CTLA*-4 expression did not differ for AA, AG and GG genotypes of exon 1 +49A/G polymorphism in vitiligo patients as compared to controls (p=0.877, p=0.437 and p=0.360 respectively). However, the expression levels of both fl*CTLA*-4 and s*CTLA*-4 were differed significantly for GG genotype of CT60A/G polymorphism in vitiligo patients as compared to controls (p=0.004 and p=0.005 respectively). The AA genotype did not differ for fl*CTLA*-4 and s*CTLA*-4 expression levels in patients and controls (p=0.343 and p=0.205 respectively).

Further, the expression levels of both fl*CTLA*-4 and s*CTLA*-4 were analyzed with respect to haplotypes generated from the two investigated polymorphisms of *CTLA*-4. Both fl*CTLA*-4 and s*CTLA*-4 expression levels were significantly differed and

associated with AG haplotypes in patients and controls (p=0.036 and p=0.020 respectively). Other, three haplotypes: AA, GA and GG did not differ with respect to fl*CTLA*-4 and s*CTLA*-4 expression levels in patients and controls (p=0.955 & p=0.152, p=0.476 & p=0.865, p=0.075 & p=0.992 respectively).

Ratio of sCTLA-4 and flCTLA-4 mRNA expression in vitiligo patients and controls:

The expression level of s*CTLA*-4 and fl*CTLA*-4 was also analyzed as ratio of s*CTLA*-4: fl*CTLA*-4 in vitiligo patients and controls. We could not detect any significant difference in the ratio of s*CTLA*-4 to fl*CTLA*-4 mRNA expression between patients and controls (p=0.346). However, AG genotype of exon 1 +49A/G polymorphism showed significant difference for the ratio of s*CTLA*-4 and fl*CTLA*-4 mRNA expression in patients and controls (p=0.049). Whereas the other two genotypes: AA and GG did not differ for the ratio of s*CTLA*-4 and fl*CTLA*-4 mRNA expression in patients and controls (p=0.668 and p=0.964). When ratio of s*CTLA*-4 and fl*CTLA*-4 mRNA expression for CT60A/G genotypes were compared in patients and controls GG genotype showed significantly decreased ratio (p=0.019), conversely the AA genotype did not differ significantly (p=0.096). Moreover, ratio of s*CTLA*-4 and fl*CTLA*-4 mRNA expression did not differ significantly for any of the haplotypes (AA, AG, GA, GG) in patients and controls (p=0.159, p=0.068, p=0.966 and p=0.585 respectively).

Our results suggest an association between CT60A/G polymorphism and susceptibility to vitiligo. Interestingly, we found decreased mRNA expression of both fl*CTLA*-4 and s*CTLA*-4 in patients and CT60G allele greatly reduced the mRNA expression of both isoforms suggesting its crucial role in pathogenesis of vitiligo. CT60AA genotype did not modulate *CTLA*-4 mRNA expression suggesting patients harboring it may have other genetic factors involved in disease pathogenesis supporting the fact that vitiligo may have varied type of precipitating factors. Further, decreased s*CTLA*-4 mRNA levels in patients with haplotype AG (+49A:CT60G) reveals the positive correlation of CT60G in vitiligo pathogenesis.

In conclusion, our findings suggest that the dysregulated *CTLA*-4 expression in vitiligo patients could result, at least in part, from variations at the genetic level. For the first time, we show that the 3' UTR CT60A/G polymorphism of the *CTLA*-4 gene influences both full length and soluble *CTLA*-4 mRNA levels in vitiligo patients, and thus this genotype-phenotype correlation of *CTLA*-4 supports the autoimmune pathogenesis of vitiligo.

6. Role of Melanocyte proliferating gene 1 (*MYG*1) promoter and structural polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility:

*MYG*1 (Melanocyte proliferating gene 1 or Gamm1 or C12orf10, NM021640) is a ubiquitous nucleo-mitochondrial protein, involved in early developmental processes as well as in stress conditions (Kingo et al. 2006). Recently, an elevated expression of *MYG*1 mRNA has been shown in both uninvolved and involved skin in case of vitiligo (Kingo et al. 2006). The precise function of MYG1 in the development of vitiligo is not clear. However, up-regulation of several immune response-related genes after siRNA-mediated knockdown of *MYG*1 mRNA suggests that MYG1 may participate in pathways proposed by autoimmune theory of vitiligo pathogenesis. Alternatively, mitochondrially localized MYG1 can be involved in the regulation of altered metabolism and imbalance of antioxidants in vitiligo patients.

We explored -119C/G promoter polymorphism (rs1465073) and 11-12AA/GC structural polymorphism (rs1534284-rs1534283; Arg4Gln) in *MYG*1 gene to study their associations with vitiligo susceptibility and correlated them with *MYG*1 transcript levels in vitiligo patients and controls of Gujarat.

A) Genetic association of MYG1 -119C/G (rs1465073) polymorphism with vitiligo patients and controls of Gujarat:

The SNP rs1465073 is located 119 bp upstream of MYG1 translation start site (ATG) and designated as *MYG*1 promoter polymorphism (-119C/G). Previously, a strong association of -119G allele was observed in patients as compared to controls (Philips et al., 2010).

In the present study *MYG1* -119C/G promoter polymorphism (rs1465073) was genotyped in 662 patients and 724 controls. Genotype frequencies were significantly different between patients and controls suggesting a strong association of *MYG1* -119C/G promoter polymorphism with vitiligo susceptibility (p=0.001). Also, allele frequencies significantly differed between patients and controls for this polymorphism (p=0.002). Our results are in concordance with those of Philips et al., (2010) suggesting the important role of *MYG1* -119C/G promoter polymorphism in vitiligo susceptibility.

B) Genetic association of MYG1 11-12 AA/GC (rs1534284-rs1534283; Arg4Gln) polymorphism with vitiligo patients and controls of Gujarat:

This polymorphism involves nucleotides 11-12 (rs1534284-rs1534283; Arg4Gln) downstream from translation start site ATG. These nucleotides are coding second and third positions of amino acid four in the N-terminus of Myg1 protein (CAA and CGC, respectively). Amino acid four is part of a mitochondrial targeting signal (MTS) of Myg1 protein. The polymorphism is potentially functional, since it changes basic amino acid (Arginine) into polar and uncharged amino acid (Glutamine) which disturbs a common property of mitochondrial targeting sequence to form an amphiphilic helical structure that is essential for the effective transport of a mitochondrial protein.

MYG1 11-12 AA/GC (Arg4Gln) polymorphism was genotyped in 662 patients and 724 controls. This polymorphism was predominantly monogenic in patients and controls with only *MYG*1 GC (4Arg) alleles being present suggesting the non-association of this polymorphism with vitiligo susceptibility.

C) Relative gene expression of MYG1 in patients and controls:

*MYG*1 mRNA expression was assessed in 45 patients and 48 controls by real time PCR. Relative gene expression analysis of *MYG*1 showed significantly higher mRNA levels in patients as compared to controls ($p=0.004$) suggesting the crucial role of *MYG*1 in vitiligo susceptibility. The $2^{-\Delta\Delta Cp}$ analysis showed ~1 fold increased expression of *MYG*1 in patients as compared to controls.

D) Establishment of genotype-phenotype correlation with vitiligo susceptibility:

*MYG*1 mRNA levels in skin samples of healthy controls correlated with *MYG*1 promoter polymorphism -119C/G and subjects with homozygous -119G allele had significantly higher MYG1 mRNA levels than subjects with homozygous -119C allele (Philips et al., 2010). More samples will be analyzed to establish the genotype-phenotype correlation for the *MYG1* polymorphisms and the results will be shown in the thesis.

7. Role of NACHT leucine-rich repeat protein 1 (*NALP1*) gene promoter and structural polymorphisms in vitiligo pathogenesis:

*NALP*1, *(NLRP1)* encodes NACHT leucine-rich repeat protein 1, a key regulator of the innate immune system. *NALP*1 plays a role in cellular apoptosis, its over expression stimulates caspase mediated apoptosis in a variety of cell types. Variations in the *NALP*1 gene have been reported to confer risk for vitiligo and autoimmune disorders in Caucasian patients from the United Kingdom, the United States, and Romania (Jin et al., 2007).

A) Genotyping of NALP1 T/A (rs12150220), T/C (rs2670660) and G/A (rs6502867), polymorphisms in vitiligo patients and controls of Gujarat:

The T/A (rs12150220) is a non-synonymous coding SNP which substitutes leucine155 to histidine. The amino acid sequence, including Leu155, is highly conserved throughout primate evolution, suggesting that this region is critical for protein function. The T/C (rs2670660) SNP is present in *NALP*1 promoter region which is conserved in the human, chimpanzee, macaque, bush baby, cow, mouse and rat, suggesting that this variant is functionally significant. It alters predicted binding motifs for the transcription factors HMGA1 [HMG-I(Y)] and MYB. MYB regulates transcription during the differentiation, proliferation, and apoptosis of erythroid, myeloid, and lymphoid cell lineages. The G/A (rs6502867) SNP is located in an intron towards the 3' end of the *NALP*1 gene. The Romanian study confirms genetic association of generalized vitiligo with *NALP*1 SNP rs6502867 and SNP rs2670660. Genotyping of *NALP1* G/A (rs6502867), T/C (rs2670660), T/A (rs12150220) SNPs is being done in vitiligo patients and controls of Gujarat and the results will be shown in the thesis.

B) Relative gene expression of NALP1 in patients and controls:

NALP1 transcript levels will be measured in vitiligo patients and controls with *GAPDH* as a reference gene using real time PCR and the results will be shown in the thesis.

C.) Establishment of genotype-phenotype correlation with vitiligo susceptibility:

To assess the effect of *NALP*1 G/A, T/C and T/A polymorphisms, genotypes will be compared with the *NALP*1 mRNA levels between patients and controls and thus genotype-phenotype correlation will be established for vitiligo susceptibility.

8. Determination of $CD4^+/CD8^+$ ratio and estimation of $CD4^+CD25^+$FoxP3 T-regulatory cells (Tregs) in vitiligo patients and controls:

A) Determination of $CD4^+/CD8^+$ ratio *in vitiligo patients and controls of Gujarat:*

High frequencies of melanocyte-reactive cytotoxic T cells in the peripheral blood of vitiligo patients, peri-lesional T-cell infiltration and melanocyte loss *in situ* suggest the important role of cellular autoimmunity in the pathogenesis of this disease. In most vitiligo patients the balance of cytotoxic/suppressor and helper/inducer T-cells in peripheral blood is disturbed which might lead to predominance of T-cell subtypes. The ratio of $CD4^+/CD8^+$ cells will be monitored by FACS analysis in vitiligo patients and controls of Gujarat population and the results will be shown in the thesis.

B) Estimation of $CD4^+CD25^+$FoxP3 T-regulatory cells (Tregs) in vitiligo patients and controls of Gujarat:

Regulatory T cells (Tregs) are critical for the maintenance of immune cell homeostasis. Specifically, Treg cells maintain order in the immune system by enforcing a dominant negative regulation on other immune cells. CD4+CD25+ Tregs is an important component of immune system in controlling autoimmunity. These naturally occurring T cells can control actively and dominantly the activation and function of autoreactive T cells that have escaped from the thymus and can prevent development of the autoimmune disease. The FoxP3 is a special marker for Treg cells; it has been identified as a master gene for the cell-lineage commitment, development and function of Treg cells.

In the present study, vitiligo patients exhibited decreased s*CTLA*-4 and fl*CTLA*-4 transcript levels. Recently, Gerold et al., (2011) have also shown decreased s*CTLA*-4 levels in Type-1 diabetic condition suggesting that lower s*CTLA*-4 expression may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. Hence, CD4+CD25+ Tregs will be monitored in vitiligo patients and controls by FACS analysis and the results will be shown in the thesis.

Conclusion:

The present study has made an attempt to understand the pathogenesis of vitiligo in Gujarat vitiligo patients by assessing antimelanocyte antibody levels and role of selected candidate genes involved in autoimmune pathogenesis of vitiligo. Significant increase in antimelanocyte antibody levels in vitiligo patients supports the involvement of autoimmunity in vitiligo pathogenesis. Our results on genetic study suggests that candidate genes: *PTPN22*, *ACE*, *MBL2*, *IFN-γ* are not playing important role in vitiligo pathogenesis. However, significant association of polymorphisms of *CTLA-4* and *MYG1* genes and their altered mRNA expression suggest their crucial role in vitiligo susceptibility.

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