CHAPTER VI

ROLE OF TUMOR NECROSIS FACTOR α (TNFα) IN VITILIGO SUSCEPTIBILITY

6.1 INTRODUCTION:

Vitiligo is an acquired, non-contagious disease in which progressive, patchy, multifocal loss of pigmentation of skin, overlying hair, and often mucous membranes results from loss of melanocytes from the involved areas (Taieb and Picardo, 2009). It affects 0.2-1% of the world population (Spritz, 2008). In India, the incidence of vitiligo is found to be 0.5% (Das *et al.,* 1985). It is associated with increased risk of several other autoimmune diseases such as: autoimmune thyroid disease (Graves' disease and 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 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; however, recent genome-wide association studies (GWAS), have identified generalized vitiligo susceptibility genes which almost universally involve immune regulation and immune targeting of melanocytes, that have led to the general consensus that generalized vitiligo is a primary autoimmune disease, though 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, GCH1, MBL2, PTPN22,* and *VDR* (Das *et al.,* 1985; Spritz, 2007). 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, PTPN22, NALP1, XBP1, FOXP1, IL2RA have* been tested for genetic association with vitiligo (Spritz, 2010).
Cytokines are important mediators of immunity and their response due to imbalance or deficiency in the cytokine network may largely determine autoimmune disease susceptibility and severity. Tumor necrosis factor (TNF)α is a multifunctional, proinflammatory cytokine which plays an important role in several autoimmune diseases like rheumatoid arthritis, pernicious anemia, diabetes mellitus etc.

TNFα plays an important role in apoptosis through activation of the receptor-mediated apoptosis pathway in numerous cell types (Gupta and Gollapudi, 2006). It is produced by many different cell types, including activated T cells, fibroblasts, adipocytes, smooth muscle cells and keratinocytes. In the epidermal melanin unit of epidermis, a melanocyte is in close interaction with ~32 keratinocytes. The keratinocytes synthesize cytokines, such as TNFα, interleukin (IL) 1α, IL-6, and transforming growth factorβ (TGFβ), which are paracrine inhibitors of human melanocyte proliferation and melanogenesis. TNFα also affects the apoptotic pathway of melanocytes and its level may play an important role in vitiligo pathogenesis. Moreover, TNFα can inhibit melanocyte stem cell differentiation (Alghamdi *et al.,* 2012; 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).

TNFA gene locus is located within the Class III region of the human major histocompatibility complex (MHC) on chromosome 6 (6p21.31) spanning about 3 kb and contains 4 exons. Regulation of TNFα production occurs at both the transcriptional and post-transcriptional levels, with regulatory sequences within the 5' end of the gene controlling the rate of transcription (Spriggs *et al.,* 1992). Several single-nucleotide polymorphisms (SNPs) have been identified in the human *TNFA* gene promoter region having the potential to cause structural changes within regulatory sites that could affect the function or régulation of TNFα production. The location of its gene within major histocompatibility complex and biological activities has raised the possibility that polymorphisms within this locus may contribute to the pathogenesis of wide range of autoimmune and infectious diseases.

The promoter polymorphisms at positions: -238, -308, -857, and -1031 may lead to a higher rate of *TNFA* gene transcription whereas -863 leads to decrease the transcription. These polymorphisms combined could contribute to the autoimmune process making it an ideal candidate for the development of vitiligo.

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

i.) To determine whether the promoter polymorphisms of *TNFA* [-238 (G/A; rs361525), -308 (G/A; rs1800629), -857 (C/T; rs1799724), -863 (C/A; rs1800630) and -1031 (T/C; rs1799964)] are associated with vitiligo susceptibility and modulate *TNFα* transcript and protein levels.

ii.) To measure and compare *TNFA* and *ICAM1* transcript and serum TNFα levels in patients with vitiligo and in unaffected controls.

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

6.2 MATERIALS AND METHODS

6.2.1 Study Subjects:

The study group included 977 vitiligo patients [733 generalized (including acrofacial vitiligo and vitiligo universalis) and 244 localized vitiligo cases] comprised of 451 males and 526 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 990 ethnically and sex-matched unaffected individuals (447 males and 543 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.

6.2.2 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.

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

	Vitiligo Patients	**Controls**
	(n = 977)	(n = 990)
Average age (mean age ± SD)	32.45 ± 13.48 yrs	28.23 ± 14.42 yrs
Sex: male	451 (46.16%)	447 (45.15%)
female	526 (53.84%)	543 (54.85%)
Age of onset (mean age ± SD)	21.25 ± 12.53 yrs	NA
Duration of disease (mean ± SD)	7.8 ± 6.9 yrs	NA
Type of vitiligo		
Generalized	733 (75.03%)	NA
Localized.	244 (24.97%)	NA
Active vitiligo	682 (69.81%)	NA
Stable vitiligo	295 (30.19%)	NA

6.2.3 Genotyping of *TNFA* promoter polymorphisms:

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to genotype all five promoter polymorphisms of *TNFA* gene (Figure 1). 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 (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) used were: *Bam*HI, *Nco*I, *Tai*I and *Bbs*I for digesting amplicons of -238 G/A, -308 G/A, -857 C/T, -863 C/A and -1031 T/C of *TNFA* 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 as per the manufacturer's instruction. The digestion products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 3.5% agarose gels or 15% polyacrylamide 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).

6.2.4 Determination of *TNFA, ICAM1* and *GAPDH* mRNA expression:

6.2.4.1 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/ 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).

6.2.4.2 Real-time PCR:

The expression of *TNFA, 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, 63°C for 30 s, 72°C for 30 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melt curve analysis was carried out on the product formed (Figure 7). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

Table 2. Primers for *TNFA* promoter SNPs genotyping and gene expression analysis.

Gene/SNP* Primer	Sequence (5' to 3')	Annealing Temp. (°C)	Product size (bp)	Restriction Enzyme (Digested Products)
(rs361525)				*Bam*HI
TNFA -238G/A F	CTGTCCCAGGCTTGTCCTGCTAC	66	376	(352bp &
TNFA -238G/A R	CTCACACTCCCCATCCTCCCGGATC			24bp)
(rs1800629)				*Nco*I
TNFA -308G/A F	GAGGCAATAGGTTTTGAGGGCCAT	57	360	(339bp &
TNFA -308G/A R	TCTGCTGTCCTTGCTGAGGGA			21bp)
(rs1799724)				*Tai*I
TNFA -857C/T F	GCATCTGCACCCTCGATGAAG	58	325	(306bp &
TNFA -857C/T R	CCTCTACATGGCCCTGTCTAC			19bp)
(rs1800630)				*Tai*I
TNFA -863C/A F	GCTCAAAGGGAGCAAGAGCTG	65	323	(302bp &
TNFA -863C/A R	CTACATGGCCCTGTCTTCGTTACG			21 bp)
(rs1799964)				*Bbs*I
TNFA -1031T/C F	GCTCAAAGGGAGCAAGAGCTG	66	481	(313bp &
TNFA-1031T/C R	GCTGGTTTCAGTCTTGGCTTCC			168bp)
TNFA gene expression F	GCCCCCAGAGGGAAGAGTTCCCCA	65	124	-
TNFA gene expression R	GCTTGAGGGTTTGCTACAACATGGGC			
ICAM1 gene expression F	TCTGTTCCCAGGACCTGGCAATG	65	282	-
ICAM1 gene expression R	GGAGTCCAGTACACGGTGAGGAAG			
GAPDH gene expression F	CATCACCATCTTCCAGGAGCGAG	65	122	-
GAPDH gene expression R	CCTGCAAATGAGCCCCAGCCT			

*The nucleotide change was from the ancestral (major) to the derived (minor) allele.

Bold letters within the primer sequences represent a forced mismatch.

6.2.5 Estimation of serum TNFα levels by enzyme-linked immunosorbent assay:

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

6.2.6 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all five polymorphisms of *TNFA* 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 *TNFA* promoter 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). *p*-values less than 0.01 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 and He, 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). Relative gene expression of *TNFA* and serum TNFα 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).

6.3 RESULTS

6.3.1 Association of *TNFA* promoter polymorphisms with generalized vitiligo:

The genotype and allele frequencies of the investigated *TNFA* promoter polymorphisms in 733 generalized vitiligo patients and 990 controls are summarized in Table 3. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations in both patient and control groups (p>0·05).

The five promoter polymorphisms of *TNFA* were found to be in significant association with generalized vitiligo patients (p<0.0001) when genotypes were compared using chi-square test-3x2 contingency table with Bonferroni's correction for multiple testing (Table 3). Also, there was significant difference in allele frequencies of these polymorphisms between generalized patients and controls when compared with 2x2 contingency table (p<0.0001) (Table 3). Interestingly, -238A and -308A alleles were found to increase the risk of generalized vitiligo by 6.35 and 4.326 fold respectively [odds ratio (OR): 6.35; 95% confidence interval (CI): (5.320-7.590); odds ratio (OR): 4.326; 95% confidence interval (CI): (3.623-5.165)] (Table 3). However, -857T, -863A and -1031C alleles were found to increase the risk of generalized vitiligo by 2.181, 2.231 and 1.960 fold respectively [odds ratio (OR): 2.181; 95% confidence interval (CI): (1.885-2.524); odds ratio (OR): 2.231; 95% confidence interval (CI): (1.894-2.629); odds ratio (OR): 1.960; 95% confidence interval (CI): (1.675-2.294)] (Table 3). This study has 95.0% statistical power for the effect size 0.08 to detect association of *TNFA* promoter polymorphisms at p<0.05 in generalized vitiligo patients and control population.

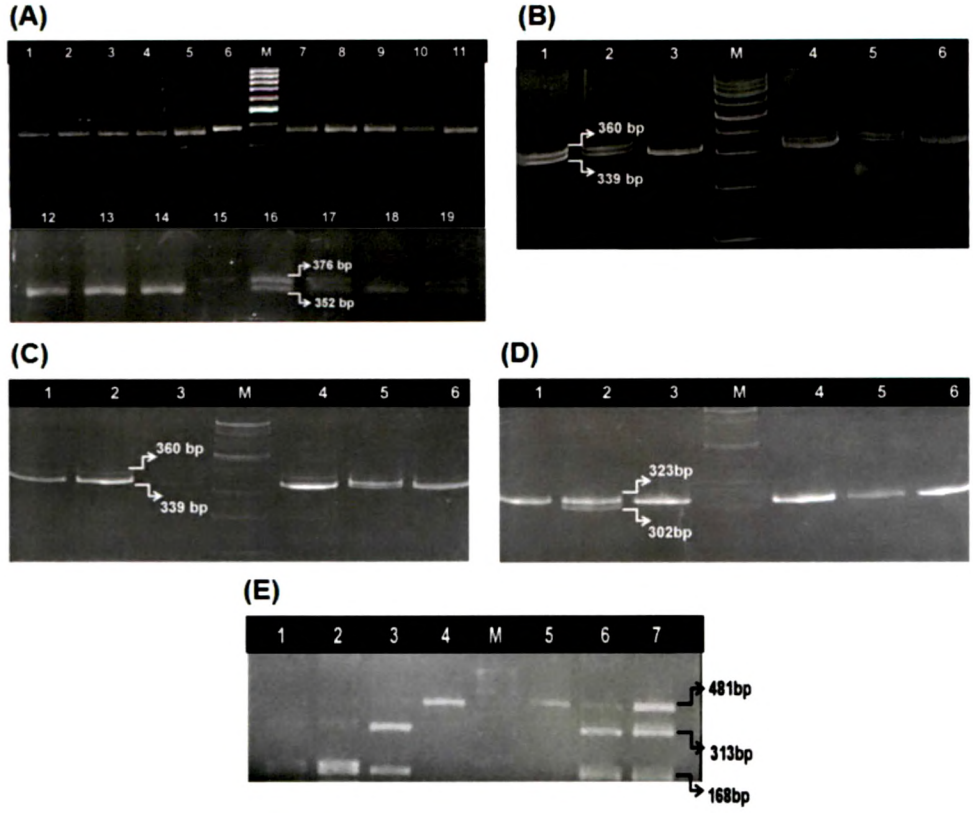

Figure 1. (A) PCR-RFLP analysis of *TNFA* -238 G/A polymorphism on 3.5 % agarose gel: lanes: 1, 2, 3, 4, 5, 11, 12, 13, 14 & 18 show homozygous (GG) genotypes; lanes: 16, 17 & 19 show heterozygous (GA) genotypes; lanes: 6, 7, 8, 9 10 & 15 show homozygous (AA) genotypes; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *TNFA* -308 G/A polymorphism on 10% polyacrylamide gel: lanes: 1, 2, 4 & 5 show heterozygous (GA) genotypes; lanes: 3 & 6 show homozygous (GG) genotypes; lane: 11 shows homozygous (AA) genotype; lane M shows 100 bp DNA ladder.

(C) PCR-RFLP analysis of *TNFA* -857 C/T polymorphism on 10% polyacrylamide gel: lanes: 1, 2, 4, 5 & 6 show heterozygous (CT) genotypes; lane: 3 shows homozygous (GG) genotype; lane M shows 100 bp DNA ladder.

(D) PCR-RFLP analysis of *TNFA* -863 C/A polymorphism on 10% polyacrylamide gel: lanes: 1, 3, 4, 5 & 6 show homozygous (CC) genotypes; lane: 2 shows heterozygous (CA) genotype; lane M shows 100 bp DNA ladder.

(E) PCR-RFLP analysis of *TNFA* -1031 T/C polymorphism on 2.0 % agarose gel: lanes: 4 & 5 show homozygous (TT) genotypes; lanes: 6 & 7 show heterozygous (TC) genotypes; lanes: 1, 2 & 3 show homozygous (CC) genotype; lane M shows 100 bp DNA ladder.

Table 3. Association study for *TNFA* promoter polymorphisms in patients with generalized vitiligo from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 729)	(n = 990)		0.192	
	GG	250 (0.34)	798 (0.81)	<0.0001	(P)	
rs361525	GA	339 (0.47)	178 (0.18)			
(-238;	AA	140 (0.19)	14 (0.01)		0.263	
G/A)	Allele				(C)	6.35
	G	839 (0.58)	1774(0.90)	<0.0001		(5.320-
	A	619 (0.42)	206 (0.10)			7.590)
	Genotype	(n = 728)	(n = 981)		0.093	
	GG	317 (0.44)	780 (0.80)		(P)	
rs1800629	GA	311 (0.43)	184 (0.19)	<0.0001		
(-308;	AA	100 (0.13)	17 (0.01)		0.114	
G/A)	Allele				(C)	4.326
	G	945 (0.65)	1744 (0.89)	<0.0001		(3.623-
	A	511 (0.35)	218 (0.11)			5.165)
	Genotype	(n = 728)	(n = 984)		0.563	
	CC	249 (0.34)	563 (0.57)		(P)	
rs1799724	CT	347 (0.48)	352 (0.36)	<0.0001		
(-857;	TT	132 (0.18)	69 (0.07)		0.173	
C/T)	Allele				(C)	2.181
	C	845 (0.58)	1478 (0.75)	<0.0001		(1.885-
	T	611 (0.42)	490 (0.25)			2.524)
	Genotype	(n = 728)	(n = 984)		0.084	
	CC	365 (0.50)	698 (0.71)		(P)	
rs1800630	CA	287 (0.40)	253 (0.26)	<0.0001		
(-863;	AA	76 (0.10)	33 (0.03)		0.094	
C/A)	Allele				(C)	
	C	1017(0.70)	1649 (0.84)	<0.0001		2.231
	A	439 (0.30)	319 (0.16)			(1.894-
						2.629)
	Genotype	(n = 733)	(n = 989)		0.063	
	TT	354 (0.48)	653 (0.66)		(P)	
rs1799964	TC	295 (0.40)	295 (0.30)	<0.0001		
(-1031;	CC	84 (0.12)	41 (0.04)		0.296	
T/C)	Allele				(C)	
	T	1003(0.68)	1601(0.81)	<0.0001		1.960
	C	463 (0.32)	377 (0.19)			(1.675-
						2.294)

'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,
Statistical significance was considered at p value ≤ 0.01 due to Bonferroni's correction for multiple testing.

6.3.2 Association of *TNFA* promoter polymorphisms with localized vitiligo:

The genotype and allele frequencies of the investigated *TNFA* promoter polymorphisms in 244 localized vitiligo patients and 990 controls are summarized in Table 4. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations in both patient and control groups (p>0·05) except for -238G/A and -863C/A in patients (p=0.0001 and p=0.014 respectively).

The five promoter polymorphisms of *TNFA* were found to be in significant association with localized vitiligo patients (p<0.0001) when genotypes were compared using chi-square test-3x2 contingency table with Bonferroni's correction for multiple testing (Table 4). Also, there was significant difference in allele frequencies of these polymorphisms between localized patients and controls when compared with 2x2 contingency table (p<0.0001) (Table 4). Although, all five promoter polymorphisms of *TNFA* were found to be associated with localized vitiligo patients none of the susceptible alleles of these polymorphisms were found to be a risk for localized vitiligo as suggested by the odds ratio (Table 4). This study has 88.0% statistical power for the effect size 0.08 to detect association of *TNFA* promoter polymorphisms at p<0.05 in localized vitiligo patients and control population.

Table 4. Association study for *TNFA* promoter polymorphisms in patients with localized vitiligo from Gujarat.

SNP	Genotype or allele	Localized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 241)	(n = 990)		0.0001	
	GG	75 (0.31)	798(0.81)		(P)	
rs361525	GA	91 (0.38)	178(0.18)	<0.0001		
(-238;	AA	75 (0.31)	14 (0.01)		0.263	
G/A)	Allele				(C)	0.116
	G	241 (0.50)	1774(0.90)	<0.0001		(0.092-
	A	241 (0.50)	206(0.10)			0.146)
	Genotype	(n = 241)	(n = 981)		0.278	
	GG	79 (0.33)	780 (0.80)		(P)	
rs1800629	GA	125 (0.52)	184 (0.19)	<0.0001		
(-308;	AA	37 (0.15)	17 (0.01)		0.114	
G/A)	Allele				(C)	0.178
	G	283 (0.59)	1744 (0.89)	<0.0001		(0.141-
	A	199 (0.41)	218 (0.11)			0.224)
	Genotype	(n = 241)	(n = 984)		0.864	
rs1799724	CC	86 (0.36)	563 (0.57)		(P)	
(-857;	CT	117 (0.49)	352 (0.36)	<0.0001		
C/T)	TT	38 (0.15)	69 (0.07)		0.173	
	Allele				(C)	0.496
	C	289 (0.60)	1478 (0.75)	<0.0001		(0.403-
	T	193 (0.40)	490 (0.25)			0.612)
	Genotype	(n = 242)	(n = 984)		0.014	
rs1800630	CC	137 (0.57)	698 (0.71)		(P)	
(-863;	CA	80 (0.33)	253 (0.26)	<0.0001		
C/A)	AA	25 (0.10)	33 (0.03)		0.094	
	Allele				(C)	
	C	354 (0.73)	1649 (0.84)	<0.0001		0.527
	A	130 (0.27)	319 (0.16)			(0.417-
						0.666)
	Genotype	(n = 244)	(n = 989)		0.607	
rs1799964	TT	112 (0.46)	653 (0.66)		(P)	
(-1031;	TC	104 (0.43)	295 (0.30)	<0.0001		
T/C)	CC	28 (0.11)	41 (0.04)		0.296	
	Allele				(C)	0.483
	T	328 (0.67)	1601(0.81)	<0.0001		(0.388-
	C	160 (0.33)	377 (0.19)			0.601)

'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,

Statistical significance was considered at p value ≤ 0.01 due to Bonferroni's correction for multiple testing.

6.3.3 Linkage disequilibrium (LD) and haplotype analyses of *TNFA* promoter polymorphisms:

The LD analysis revealed that the five promoter polymorphisms investigated in the *TNFA* gene were in low to moderate LD association in both generalized as well as localized vitiligo patients (Figures 2A & B). In particular, -238G/A and -308G/A polymorphisms were in moderate LD association with D'= 0.485 and 0.484 in generalized and localized vitiligo patients respectively (Tables 5 & 6).

A haplotype evaluation of the five polymorphic sites was performed and the estimated frequencies of the haplotypes were differed significantly between generalized vitiligo patients and controls (global $p<0.0001$). Also, localized vitiligo patients exhibited significantly different frequencies of haplotypes as compared to controls (global $p<0.0001$) (Tables 7 & 8).

The susceptible haplotypes: AACAT, AACCT, AATCC, AATCT and AGCCT were more frequently observed in generalized vitiligo patients as compared to controls and were found to increase the risk of generalized vitiligo as suggested by odds ratio (Table 7). However, the non-susceptible haplotypes: GGCAT, GGCCC, GGCCT, GGTCC, GGTCT were more frequently observed in controls as compared to generalized vitiligo patients (Table 7).

Furthermore, susceptible haplotypes: AACAT, AACCT, AATCC, AATCT, AGCCC and AGCCT were more frequently observed in localized vitiligo patients as compared to controls and were found to increase the risk of localized vitiligo as suggested by odds ratio (Table 8); however, the non-susceptible haplotypes: GGCAT, GGCCC, GGCCT, GGTAT and GGTCC were more frequently observed in controls as compared to localized vitiligo patients (Table 8).

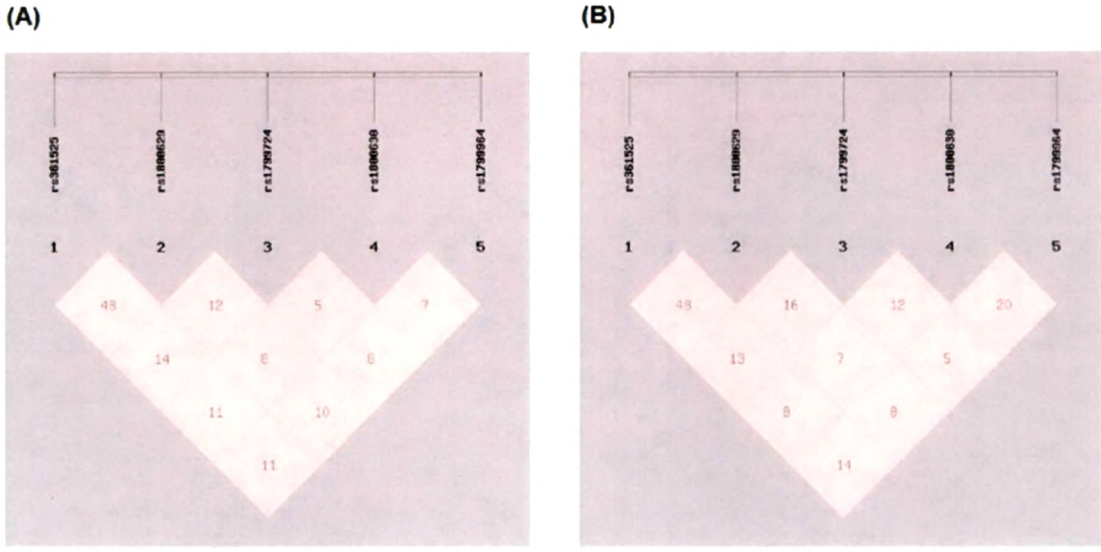

Figure 2. (A) Linkage disequilibrium (D') among *TNFA* promoter SNPs in generalized vitiligo patients and controls from Gujarat population.

(B) Linkage disequilibrium (D') among *TNFA* promoter SNPs in localized vitiligo patients and controls from Gujarat population.

Table 5. Pairwise linkage disequilibrium (D') values between *TNFA* SNPs with >3% minor allele frequencies within generalized vitiligo patients and controls from Gujarat population.

	rs1800629 (-308 G/A)	**rs1799724 (-857 C/T)**	**rs1800630 (-863 C/A)**	**rs1799964 -1031 T/C**
rs361525 (-238 G/A)	**0.485**	0.138	0.106	0.106
rs1800629 (-308 G/A)	-	0.123	0.080	0.100
rs1799724 (-857 C/T)	-	-	0.052	0.075
rs1800630 (-863 C/A)	-	-	-	0.074

*Bold value represents moderate LD.

Table 6. Pairwise linkage disequilibrium (D') values between *TNFA* SNPs with >3% minor allele frequencies within localized vitiligo patients and controls from Gujarat population.

	rs1800629 (-308 G/A)	**rs1799724 (-857 C/T)**	**rs1800630 (-863 C/A)**	**rs1799964 -1031 T/C**
rs361525 (-238 G/A)	**0.484**	0.131	0.083	0.135
rs1800629 (-308 G/A)	-	0.159	0.073	0.076
rs1799724 (-857 C/T)	-	-	0.123	0.052
rs1800630 (-863 C/A)	-	-	-	0.200

*Bold value represents moderate LD.

Table 7. Distribution of haplotypes frequencies for *TNFA* promoter polymorphisms among generalized vitiligo patients and controls.

Haplotype (-238G/A, -308 G/A, -857 C/T, -863 C/A and -1031 T/C)	**Generalized Vitiligo Patients (Freq. %) (n=1454)**	**Controls (Freq. %) (n=1936)**	***p* for Association**	***p*(global)**	**Odds ratio (95% CI)**
A A C A T	49.92 (0.034)	13.88 (0.007)	2.33e-012	<0.0001	6.547 [3.593~11.929]
A A C C T	94.79 (0.065)	21.40 (0.011)	5.67e-025		8.420 [5.232~13.551]
A A T C C	60.38 (0.042)	2.04 (0.001)	1.48e-023		54.818 [13.554~221.703]
A A T C T	71.17 (0.049)	13.93 (0.007)	4.31e-020		9.511 [5.326~16.982]
A G C C T	60.72 (0.042)	68.55 (0.035)	0.010890		1.580 [1.108~2.251]
G A C C T	31.38 (0.022)	83.64 (0.043)	0.036942		0.643 [0.423~0.977]
G G C A T	83.30 (0.057)	181.62 (0.094)	0.075190		0.782 [0.596~1.026]
G G C C C	72.25 (0.050)	206.78 (0.107)	0.000113		0.579 [0.438~0.766]
G G C C T	218.00 (0.150)	778.39 (0.402)	7.54e-033		0.347 [0.291~0.414]
G G T A T	65.08 (0.045)	51.56 (0.027)	9.25e-006		2.284 [1.571~3.320]
G G T C C	56.39 (0.039)	81.71 (0.042)	0.271883		1.215 [0.858~1.722]
G G T C T	144.86 (0.100)	254.60 (0.132)	0.934445		0.991 [0.795~1.235]

CI represents Confidence Interval,

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

Table 8. Distribution of haplotypes frequencies for *TNFA* promoter polymorphisms among localized vitiligo patients and controls.

Haplotype (-238G/A,-308 G/A, -857 C/T, -863 C/A and -1031 T/C)	Localized Vitiligo Patients (Freq. %) (n=482)	Controls (Freq. %) (n=1936)	*p* for Association	$p_{(global)}$	Odds ratio (95% CI)
A A C A T	22.81 (0.047)	13.88 (0.007)	9.51e-012	<0.0001	7.520 [3.824~14.787]
A A C C T	40.56 (0.084)	21.40 (0.011)	9.52e-022		9.047 [5.296~15.453]
A A T C C	37.87 (0.079)	2.04 (0.001)	6.66e-016		88.928 [21.64~365.32]
A A T C T	33.24 (0.069)	13.93 (0.007)	2.89e-020		11.220 [5.944~21.180]
A G C C C	20.19 (0.042)	21.79 (0.011)	7.93e-007		4.193 [2.267~7.758]
A G C C T	17.72 (0.037)	68.55 (0.035)	0.648433		1.132 [0.664~1.930]
G A C C T	5.60 (0.012)	83.64 (0.043)	0.002174		0.282 [0.119~0.668]
G G C A C	20.16 (0.042)	20.87 (0.011)	4.39e-007		4.373 [2.348~8.144]
G G C A T	21.90 (0.045)	181.62(0.094)	0.002400		0.499 [0.316~0.788]
G G C C C	19.17 (0.040)	206.78(0.107)	3.43e-005		0.375 [0.232~0.606]
G G C C T	74.89 (0.155)	778.39(0.402)	3.35e-021		0.291 [0.223~0.380]
G G T A T	15.29 (0.032)	51.56 (0.027)	0.369915		1.304 [0.729~2.330]
G G T C C	17.54 (0.036)	81.71(0.042)	0.795328		0.933 [0.550~1.580]
G G T C T	51.54 (0.107)	254.60(0.132)	0.375415		0.865 [0.628~1.192]

CI represents Confidence Interval,
(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

6.3.4 Age of onset of vitiligo and *TNFA* promoter haplotypes in patients with vitiligo:

When age of onset of the disease was correlated with the *TNFA* promoter haplotypes, patients with AACAT, AACCT, AATCC and AATCT haplotypes showed early onset of the disease as compared to GGCAT, GGCCT, GGTCC and GGTCT (p=0.001, p=0.0004, p<0.0001 and p=0.005 respectively) (Figure 3A). Patients with haplotype AATCC had an early onset of the disease as compared to GATCC haplotype (p=0.04). Moreover, patients with haplotype AATCT showed early onset of the disease as compared to AGTCT and GATCT haplotypes (p=0.001 and p=0.025 respectively) (Figure 3A). Also, patients with AGCCC haplotype had an early onset of the disease as compared to GGCCC haplotype (p=0.045); however, there was no significant difference in age of onset of the disease for haplotype AGCCT as compared to GACCT and GGCCT haplotypes (p=0.147 and p=0.481 respectively) (Figure 3A). Patients with haplotypes GGTCT and GGCCC showed no significant difference in age of onset of the disease as compared to GGTAT and GGCAC haplotypes (p=0.248 and p=0.582 respectively) (Figure 3A). 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 3B).

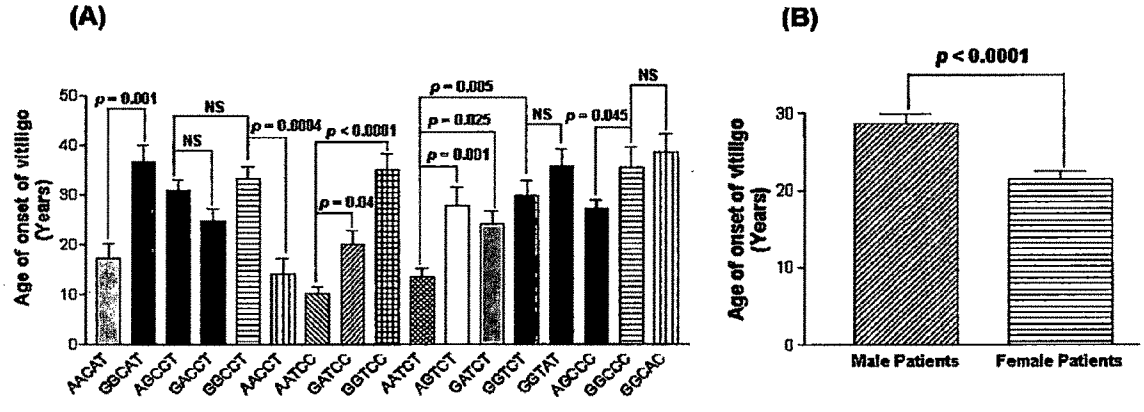

Figure 3. Age of onset of the disease in vitiligo patients.

(A) Comparison of age of onset of the disease (years) with respect to *TNFA* promoter haplotypes in 977 vitiligo patients.

(B) Comparison of age of onset of the disease (years) with respect to gender differences in 451 male patients and 526 female patients with vitiligo.

6.3.5 Relative gene expression of *TNFA* in patients with vitiligo and controls:

Comparison of the findings showed significantly increased expression of *TNFA* transcripts in 157 vitiligo patients than in 174 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.0005) (Figure 4A). Moreover, generalized vitiligo patients showed significant higher expression of *TNFA* transcripts as compared to localized vitiligo patients (p=0.0295) (Figure 4A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.445 fold change in the expression of *TNFA* transcript in patients as compared to controls (Figure 4E).

Further, the expression levels of *TNFA* were analyzed with respect to haplotypes generated from the five investigated promoter polymorphisms of *TNFA* (Figure 4B). Interestingly, *TNFA* expression was significantly increased for the haplotypes: GATCT, GATCC, AATCC and AACCT in vitiligo patients as compared to controls (p=0.013, p=0.006, p=0.023 and p=0.004 respectively); however, no significant difference was observed in *TNFA* expression for the haplotypes: GGTCC, GGCAC, GGCCT and GGTCT (p=0.517, p=0.258, p=0.790 and p=0.456 respectively).

In addition, we analyzed the *TNFA* expression based on the progression of the disease i.e. active vitiligo and stable vitiligo (Figure 4C). Active vitiligo patients showed significantly increased expression of *TNFα* transcripts as compared to the patients with stable vitiligo (p<0.0001). To check the susceptibility of the disease based on the gender differences *TNFA* expression was analyzed for male and female vitiligo patients. Female patients with vitiligo showed significantly higher *TNFA* expression as compared to male patients (p=0.0073) (Figure 4D).

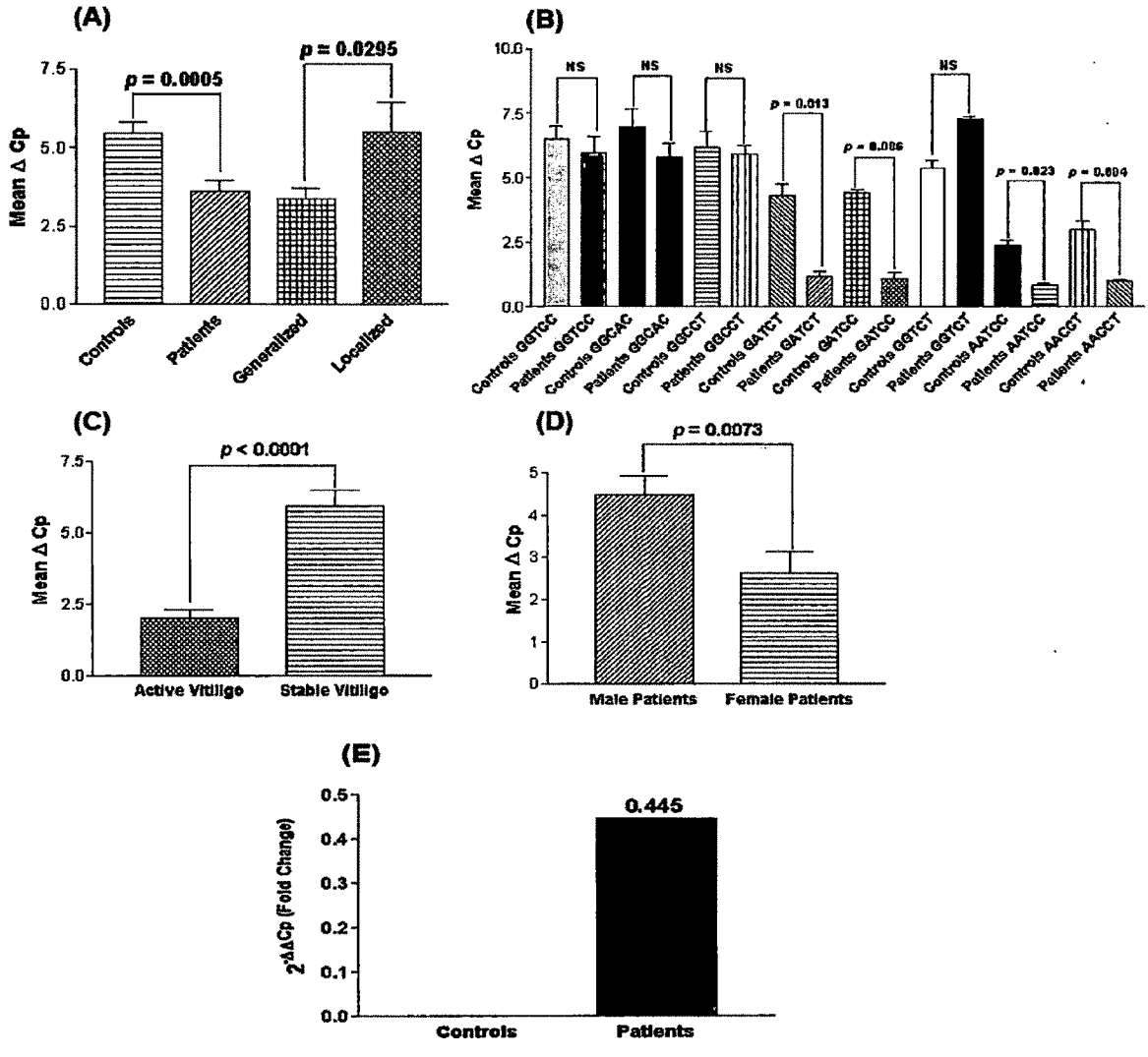

Figure 4. Relative gene expression of *TNFA* in controls and vitiligo patients.

(A) Expression of *TNFA* transcripts in 174 controls, 157 vitiligo patients, 115 generalized vitiligo patients and 42 localized vitiligo patients, as suggested by Mean ΔCp.

(B) Expression of *TNFA* transcripts with respect to *TNFα* promoter haplotypes in 157 vitiligo patients and 174 controls, as suggested by Mean ΔCp.

(C) Expression of *TNFA* transcripts with respect to activity of the disease in 108 patients with active vitiligo and 49 patients with stable vitiligo, as suggested by Mean ΔCp.

(D) Expression of *TNFA* transcripts with respect to gender differences in 68 male patients and 89 female patients with vitiligo, as suggested by Mean ΔCp.

(E) Expression fold change of *TNFA* transcripts in 157 vitiligo patients against 174 controls showed 0.445 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

6.3.6 Functional correlation of *TNFA* promoter polymorphisms with its levels in the serum:

To find any functional correlation of the investigated *TNFA* promoter polymorphisms with its level in the serum, TNFα levels were measured in 214 vitiligo patients and 236 unaffected controls. Vitiligo patients showed significant increased serum TNFα (sTNFα) levels as compared to controls (p=0.0003) (Figure 5A). Moreover, when the patient subgroups were analyzed with respect to sTNFα levels, patients with generalized vitiligo had significantly higher sTNFα levels as compared to localized vitiligo (p=0.014) (Figure 5A).

In vitiligo patients, the *TNFA* haplotypes: GATCC, AGTCT, AGCCT and AACCT were found to increase sTNFα levels (p=0.031, p=0.003, p=0.009 and p=0.007 respectively) with susceptible alleles (-238A, -308A, -857T and -1031C) as compared to controls (Figure 5B). However, no significant difference was observed in sTNFα levels for the haplotypes: GGCCT, GGTAT, GGCCC, and GGTCC (p=0.217, p=0.150, p=0.153 and p=0.868 respectively) (Figure 5B).

Furthermore, when haplotypes were analyzed for sTNFα levels in the patients based on the disease activity, patients with active vitiligo showed significantly higher sTNFα levels as compared to stable vitiligo (p<0.0001) (Figure 5C). Additionally, when the male and female patients were analyzed with respect to sTNFα levels, female patients had significantly higher levels of sTNFα as compared to male patients (p=0.0066) (Figure 5D).

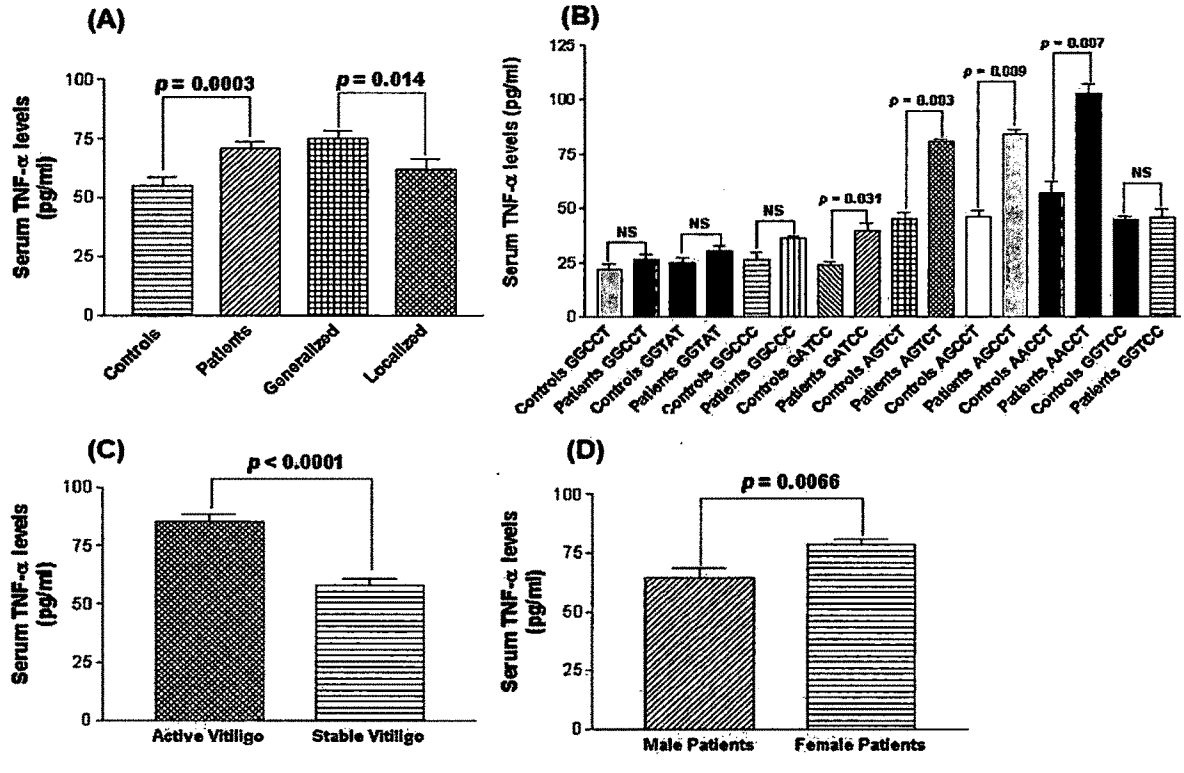

Figure 5. Serum TNFα levels in controls and vitiligo patients:

(A) Comparison of sTNFα levels (pg/ml) in 236 controls, 214 vitiligo patients, 158 generalized vitiligo patients and 56 localized vitiligo patients, as determined by ELISA.

(B). Comparison of sTNFα levels (pg/ml) with respect to *TNFA* promoter haplotypes in 214 vitiligo patients and 236 controls, as determined by ELISA.

(C) Comparison of sTNFα levels (pg/ml) with respect to activity of the disease in 150 patients with active vitiligo and 64 patients with stable vitiligo, as determined by ELISA.

(D) Comparison of sTNFα levels (pg/ml) with respect to gender differences in 97 male patients and 117 female patients with vitiligo, as determined by ELISA.

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

Comparison of the findings showed significant increase in expression of *ICAM1* transcripts in 166 vitiligo patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.008) (Figure 6A). Generalized vitiligo patients showed significantly increased mRNA levels of *ICAM1* as compared to localized patients (p=0.002) (Figure 6A). 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 6B).

In addition, the effect of *ICAM1* expression on progression of the disease i.e. active and stable cases (Figure 6C) 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 6D). 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 6E).

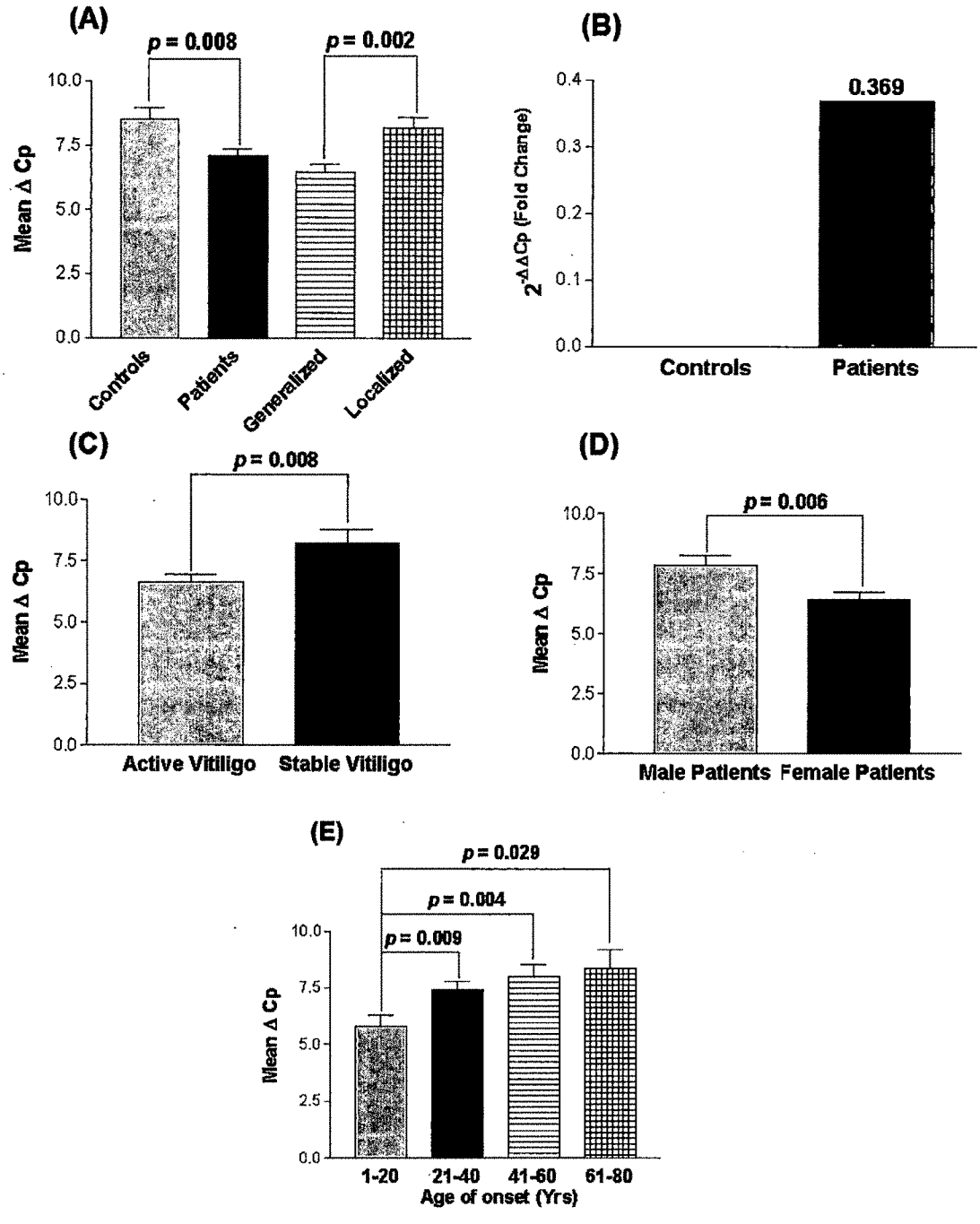

Figure 6. Relative gene expression of *ICAM1* in controls and vitiligo patients:

(A) Expression of *ICAM1* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *ICAM1* as compared to controls (p=0.008). Generalized vitiligo patients showed significantly increased mRNA levels of *ICAM1* as compared to localized patients (p=0.002).

(B) Expression fold change of *ICAM1* transcripts in 166 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 121 patients with active vitiligo and 45 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 (p=0.008).

(D) Expression of *ICAM1* transcripts with respect to gender differences in 87 male patients and 79 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 (p=0.006).

(E) Expression of *ICAM1* transcripts with respect to different age groups in 166 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 (p=0.009), 41-60 (p=0.004) and 61-80 yrs (p=0.029).

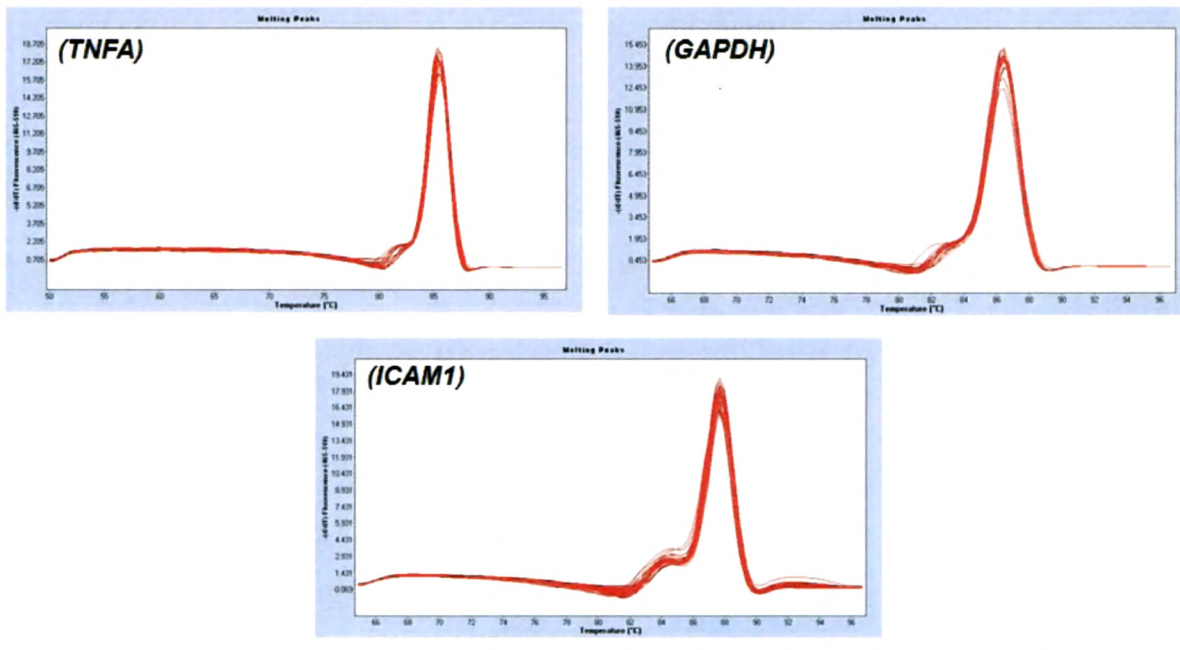

Figure 7. Melt curve analysis of *TNFA*, *ICAM1* and *GAPDH* showing specific amplification.

6.4 DISCUSSION

Vitiligo susceptibility is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Nordlund, 1997; Kim *et al.*, 1998). Our 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). Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo. 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). We have shown that 75% of vitiligo patients possessed anti-melanocyte antibodies in their circulation as compared to control population.

Association of Major Histocompatibility Complex (MHC) alleles with the 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. *TNFA* is located in *MHC* region and has strong linkage disequilibrium with *HLA* alleles. Our recent study has 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 (Singh *et al.*, 2012). Moreover, the study 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). Moreover, our recent study confirms genetic association of generalized vitiligo with SNPs in the MHC class II region in the Indian subcontinent. We have also 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 MHC region in which *TNFA* and *TNFB* are also located.

The genotype-phenotype correlation of *CTLA4* and *IL4* gene polymorphisms also supported the autoimmune pathogenesis of vitiligo in Gujarat population (Dwivedi *et*

al., 2011; Imran *et al.*, 2012) whereas our earlier studies on *MBL2, ACE, PTPN22* polymorphisms did not show significant association (Dwivedi *et al.*, 2009; Laddha *et al.*, 2008).

Cytokines are important mediators of immunity and there is now convincing evidence that cytokines also have an important role in the pathogenesis of autoimmunity (Feldmann *et al.*, 1998). The cytokines mRNA and protein levels depend on both genetic and environmental factors. Analysis of cytokine gene polymorphisms would be able to detect genetic abnormality of cytokine regulation and hence establishment of genotype-phenotype correlation may be important in unraveling the disease pathogenesis. The promoter polymorphisms of *TNFA* are reported to be involved in modulating expression of *TNFA* gene which may be responsible for melanocyte death. TNFα, is an important multifunctional cytokine secreted by macrophages, T-lymphocytes, fibroblasts and keratinocytes with wide-ranging biological effects of protection from infection, surveillance against tumors and stimulation of inflammatory responses. In the epidermis, the epidermal melanin unit consists of the close interaction of a melanocyte and an associated pool of keratinocytes. Close relationship between these two cell types is important for melanocyte survival and differentiation mainly as keratinocyte-derived cytokines act on melanocytes via specific receptors (Moretti *et al.*, 2002). Keratinocytes synthesize cytokines, such as TNFα, IL1α, IL6, and transforming growth factorβ (TGFβ), which are paracrine inhibitors of human melanocyte proliferation and melanogenesis (Moretti *et al.*, 2002). However, primary role of TNFα is in the regulation of immune cells and its overproduction has been implicated in a variety of human diseases including autoimmune disorders and cancer (Locksley *et al.*, 2001) *In vitro*, direct analysis of skin T cells from margins of vitiliginous skin show that polarized type-1 T cells (CD4+ and particularly CD8+), which predominantly secrete interferon (IFN)γ and TNFα are associated with the destruction of melanocytes during active vitiligo (Wajkowicz-Kalijska *et al.*, 2003). In vitiligo affected skin, a significantly higher expression of TNFα (Moretti *et al.*, 2002; Grimes *et al.*, 2004), IL-6 (Moretti *et al.*, 2002), IFNγ 32 (Grimes *et al.*, 2004) was detected compared with healthy controls and perilesional, non-lesional skin (Moretti *et al.*, 2002) indicating that cytokine imbalance plays an important role in the depigmentation process of vitiligo.

It has been reported that cytokines such as IFNγ and TNFα can initiate apoptosis and thus lead to melanocyte death in the context of autoimmunity and (Huang *et al.*, 2002). In addition, IFNγ and TNFα induce the expression of intercellular adhesion molecule-1 (ICAM-1) on the cell-surface of melanocytes (Yohn *et al.*, 1990). The increased expression of ICAM-1 on the melanocytes enhances T cell/melanocyte attachment in the skin and thereby may result in destruction of melanocytes in vitiligo (Al Badri *et al.*, 1993; Morelli *et al.*, 1993). TNFα also has the capacity to inhibit melanogenesis through an inhibitory effect on tyrosinase and tyrosinase related proteins (Martinez-Esparza *et al.*, 1998).

Thus, it becomes pertinent to study all *TNFA* promoter polymorphisms in adequate number of vitiligo patients and controls to elucidate the role of these polymorphisms in vitiligo susceptibility and to analyze the possible genotype - phenotype correlation. Here, we report that *TNFα* -238, -308, -857, -863 and -1031 promoter polymorphisms are significantly associated with Gujarat vitiligo patients. Our results clearly suggest the important role of TNFα in pathogenesis of vitiligo. Vitiligo patients showed significant increase in *TNFA* transcripts and protein levels as compared to controls suggesting that melanocyte death in patients could be triggered due to the increased TNFα levels.

For the first time we report that generalized vitiligo has significantly higher *TNFA* transcript and protein levels as compared to localized vitiligo patients which indicate involvement of autoimmunity in precipitation of generalized vitiligo. Our results also indicate that active vitiligo patients have significantly higher *TNFA* transcript and protein levels as compared to the patients with stable vitiligo which signifies the role of TNFα in disease progression. Our results also suggest that there are significantly higher transcript and protein levels of TNFα in female patients as compared to male patients. Moreover, female patients have an early onset 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; Afshan *et al.*, 2012).

TNFα and IFN-γ 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 *et al.*, 1993). The present study also showed increased expression of *ICAM1* in vitiligo patients suggesting that increased TNFα 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.

Interestingly, *TNFα* -308 G/A and -238 G/A polymorphisms were found to influence serum TNFα levels in patients with sarcoidosis of Asian Indian population (Sharma *et al.*, 2008) and our results are in line with this study. Furthermore, a genotype-phenotype study carried out on SLE patients showed increased *TNFA* transcript levels with -238 AA and GA genotypes as compared to GG genotypes 42 (Suárez *et al.*, 2005). In particular, in the present study when combined effect of various genotypes was analyzed in the form of haplotypes, AATCC haplotype was found to be the highest risk combination observed for the disease. Intrestingly it has all susceptible alleles except -863A which is reported to decrease the levels of TNFα. The -863 C/A polymorphism was associated with serum TNFα levels, carriers of the rare 'A' allele having a significantly lower TNFα levels in Swedish population (Skoog *et al.*, 1999). The -863A allele was associated with 31% lower transcriptional activity in chloramphenicol acetyltransferase (*CAT*) reporter gene studies in human hepatoblastoma (HepG2) cells (Skoog *et al.*, 1999). Moreover, the haplotype analysis revealed the degree of susceptibility to the disease as predicted by the odds ratio with generalized vitiligo: AATCC >AATCT >AACCT >AACAT >AGCCT and AATCC >AATCT >AACCT >AACAT >AGCCC for localized vitiligo. Also, the age of onset analysis of the disease suggested the haplotypes involved in the early age of onset in patients with vitiligo are those involved in high degree of susceptibility of the disease: AATCC >AATCT >AACCT >AACAT >AGCCC >AGCCT.

LD analysis suggests that *TNFA* -238 G/A & -308 G/A polymorphisms in moderate LD association as compared to the other investigated polymorphisms and are strongly associated with the disease risk in patients as suggested by the odds ratio. Moreover, the haplotype analysis showed the presence of haplotypes involving the susceptible alleles of *TNFA* -238 and -308 polymorphisms, having increased levels of TNFα in patients as compared to controls.

Kroeger *et al.* (1997) first showed that -308A allelic form gave a two-fold greater level of transcription than the -308G form in PMA-stimulated Jurkat and U937 cells suggesting that the -308 G/A polymorphism may play a role in the altered *TNFA* gene expression. The study of *TNFA* -308 G/A polymorphism in Iranian population have revealed significant association of -308A allele with vitiligo patients (Namian *et al.*, 2009) and these results are in line with our study however, a previous study of Turkish population suggested that *TNFA* -308 G/A polymorphism has no significant influence on vitiligo susceptibility (Yazici *et al.*, 2006). These contradictory reports may be because of the differences in ethnicity of the studied populations. However, both the studies involved less sample size and hence the association results needed further confirmation. Furthermore, there are no reports available on the effect of these *TNFA* promoter polymorphisms on its expression in vitiligo patients and the present study revealed the significant role of these promoter polymorphisms on the levels of TNFα which might be playing a central role in vitiligo pathogenesis.

It has been known that the ROS microenvironment decides the fate of a cell for TNFα mediated apoptosis (Kim *et al.*, 2010). Our earlier reports with other studies suggest that the high oxidative environment prevails in vitiligo patients for the melanocyte destruction (Agrawal *et al.*, 2004; Schallreuter *et al.*, 1991). The destruction of melanocytes might be due to the increased secretion of TNFα which further increases ROS and thus may lead to an early/defective apoptosis of the melanocytes via TNFα mediated pathway. The possiblity of the TNFα secretion is very high since the keratinocytes (a source of TNFα) surround these melanocytes forming a melanin epidermal unit and thus affect its proliferation and melanogenesis process.

Disturbances in TNFα metabolism have been well documented and found to be associated with several other autoimmune and infectious diseases such as rheumatoid arthritis (Elliot *et al.* 1994), systemic lupus erythematosus (Jacob *et al.*, 1990), crohn's

disease (Van Dullèmenn *et al.*, 1995), cerebral malaria (Mc Guire *et al.*, 1994) and lesihmaniasis (Carbera *et al.*, 1995). Previously North Indian and Caucasian studies revealed strong association of -308 G/A polymorphism with T1DM (Kumar *et al.*, 2007; Noble *et al.*, 2006) A study with psoriatic arthritis patients in Caucasian population for the five promoter polymorphisms suggested significant association of -238 G/A polymorphism with patients being -238 (A) variant, a significant risk factor for the disease (Rahman *et al.*, 2006). The *TNFA* -308 G/A polymorphism was significantly associated with susceptibility to asthma in patients of South Iran and with susceptibility to inflammatory bowel disease in European population (Kamali-Sarvestani *et al.*, 2007; Ferguson *et al.*, 2008). A metaanalysis study suggested that *TNFA* -238G/A and -308G/A polymorphisms might be used as biomarkers for psoriasis risk prediction (Li *et al.*, 2007). Furthermore, a study involving 22 SNPs in Caucasian patients with Graves' disease (GD) showed significant association of *TNFA* -238G/A and -308G/A polymorphisms (Simmonds *et al.*, 2004).

Simon and Burgor-Vargas (2008) described a patient with ankylosing spondylitis (AS) and vitiligo who was treated with infliximab (a chimeric monoclonal anti-TNF antibody), which resulted in gradual fading of vitiligo lesions suggesting that *TNFA* was involved in the pathogenesis of vitiligo. Alghamdi *et al.* (2012) also showed the effect of anti TNFα agents: infliximab, etanercept, and adalimumab in generalized vitiligo patients. The patients did not develop any new depigmented patches during treatment or at the six-month follow-up. These reports signify the involvement of TNFα in vitiligo pathogenesis.

In conclusion, our findings suggest that the increased TNFα levels in vitiligo patients could result, at least in part, from variations at the genetic level. For the first time, we show that the promoter polymorphisms of the *TNFα* gene influence the expression both at transcriptional as well as translational levels in vitiligo which in turn results into increased *ICAM1* expression. The study also emphasizes the influence of TNFα on the disease progression, onset of the disease and gender biasness for developing vitiligo. More detailed studies regarding role of TNFα in precipitation of vitiligo and the development of effective anti-TNFα agents may prove to be useful as preventive/ameliorative therapies.

6.5 REFERENCES

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