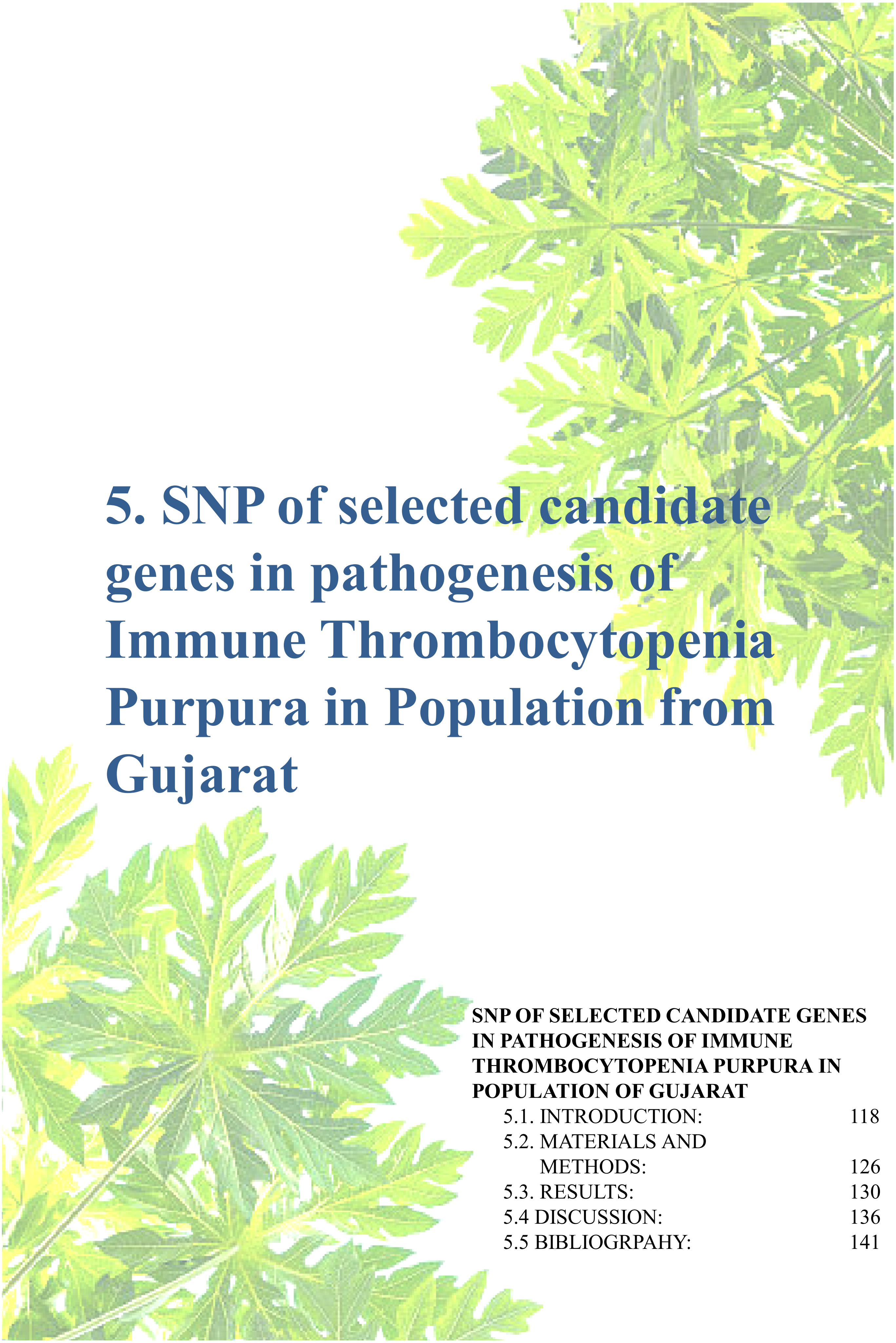


5. SNP of selected candidate genes in pathogenesis of Immune Thrombocytopenia Purpura in Population from Gujarat

SNP OF SELECTED CANDIDATE GENES IN PATHOGENESIS OF IMMUNE THROMBOCYTOPENIA PURPURA IN POPULATION OF GUJARAT

5.1. INTRODUCTION:

Platelets are the smallest component of blood and are an integral part of clot formation. Qualitatively and quantitatively normal platelets are required for hemostasis. Without adequate platelets, vascular repair after even mild trauma is inadequate and pathologic bleeding may result.

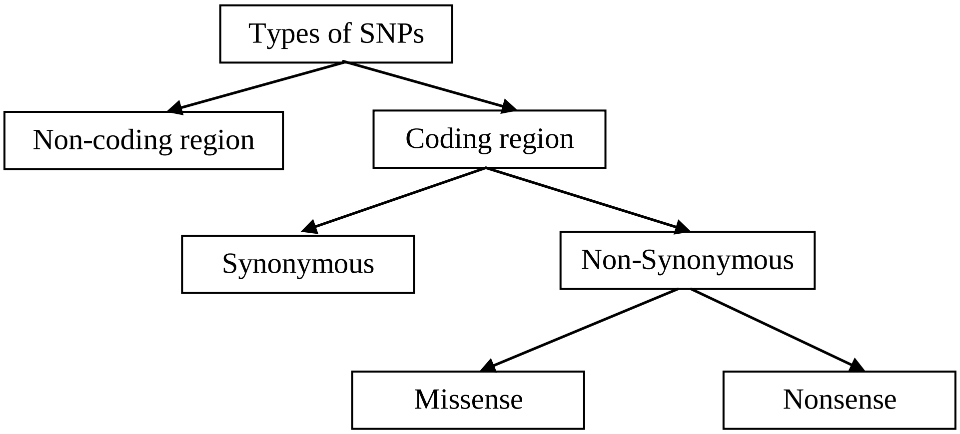
Thrombocytopenia is a disease in which the number of platelets is drastically reduced and the normal clotting mechanism of the blood is affected. Normal blood platelet count is between 150,000 to 450,000/mm^3. Some common types of Thrombocytopenia include Idiopathic Thrombocytopenic Purpura (ITP), Thrombotic Thrombocytopenic Purpura (TTP), Hemolytic-uremic Syndrome (HUS), Post transfusion purpura, Neonatal alloimmune Thrombocytopenia (NAITP), Splenic sequestration of platelets due to hypersplenism, Dengue fever, HIV-associated Thrombocytopenia and acquired Thrombocytopenia (Drug induced).

Immune Thrombocytopenic Purpura (ITP) is a common autoimmune disease, with approximately 33,000 new cases diagnosed each year in the United States (George *et al.*, 1996). Thrombocytopenia in ITP develops as a result of enhanced platelet destruction, precipitated by the interaction of autoantibodies and platelet antigens. Clinically Idiopathic Thrombocytopenic purpura (ITP) is an acquired organ-specific autoimmune disorder characterized by accelerated platelet destruction. This is due to the production of auto-antibodies against platelets, and the auto-antibodies result in the destruction of platelets via the reticuloendothelial system. The resulting thrombocytopenia is associated with a variety of hemorrhagic sequelae, including epistaxis, petechiae, gastrointestinal bleeding, and intracranial hemorrhage. The current "standard" therapies for the disease, like corticosteroid immunosuppressive therapy and spleenectomy, are associated with significant morbidity and are not effective for 25 to 30% of patients with chronic ITP (McMillan, 1997). Intravenous administration of pooled human immunoglobulin (IVIG) provides a transient increase in platelet counts for a large fraction of ITP patients; however, the high cost of this therapy prevents routine administration to individuals with chronic ITP. At present, no feasible alternative therapy is available to treat chronic ITP that is refractory to standard therapy, and fatal hemorrhage occurs in approximately 16% of affected patients (McMillan, 1997). Despite significant need for the development of new therapies for ITP, little progress has been made since the discovery of IVIG therapy in 1981. The mechanism of IVIG action are poorly understood; consequently, more specific (and perhaps less costly) therapies derived from nonhuman sources have not been developed. Clinical investigations of new ITP therapies are complicated by several factors. First, a high fraction of ITP patients (e.g., 30–40% of acute cases) experience spontaneous resolution of ITP symptoms, without therapeutic intervention (George *et al.*, 1996). Second, like other autoimmune diseases, the severity of ITP appears to wax and wane with time, as evidenced by a spontaneous oscillation of patient's platelet count. This natural fluctuation in apparent disease severity confounds the quantitative evaluation of new treatments. Third, although it is accepted that patients with very low platelet counts are more likely to experience hemorrhage, no suitable surrogate marker has been definitively associated with patient risk for severe hemorrhage. Finally, no adequate assays exist for quantification of anti-platelet antibodies (Raife *et al.*, 1997). Thus, it is impossible to evaluate treatment effects on autoantibody production or elimination.

The etiology of ITP remains unclear, but it is generally accepted that both environmental and genetic factors and probably also a synergistic relationship between these factors play important role in development of the disease. Interactions between environmental and genetic factors are proposed to explain why autoimmunity afflicts certain individuals but not others (Richardson, 2007). The environmental factors can modify the susceptibility to this disease, in part, through modulating and inducing some epigenetic changes. In recent years, epigenetics has become an exciting and evolving field of research, and its role in autoimmune diseases is extensively addressed (Richardson, 2003, 2007; Robertson, 2005)

5.1.1. SINGLE NUCEOTIDE POLYMORPHISM:

Single nucleotide polymorphisms, frequently called SNPs and pronounced “snips”. **SNP** is a variation in a single nucleotide that occurs at a specific position in the genome and is most common type of genetic variation among human population, where each variation is present to some significant degree within a population. SNPs are the cause of differences in our susceptibility to disease. A wide range of human diseases has been shown to result from SNPs (Ingram, 1956 ;Chang & Kan, 1979). The severity of illness and the way our body responds to treatments are also due to genetic variations. SNPs may be found in both coding as well as non-coding regions. SNPs that are not in protein-coding regions may still affect gene splicing, transcription factor binding, messenger RNA degradation, or the sequence of non-coding RNA. There are variations between human populations, so a SNP allele that is common in one geographical or ethnic group may be much rarer in another.



In biomedical research, SNPs' play an important role in comparing regions of the genome between cohorts (such as with matched cohorts with and without a disease). SNPs without an observable impact on the phenotype are still useful as genetic markers because of their quantity and the stable inheritance over generations (Thomas *et al.*, 2011). Some SNPs are associated with the metabolism of different drugs (Goldstein, 2001; C. R. Lee, 2004; Yanase *et al.*, 2006).

The association of a wide range of human diseases like cancer, infectious diseases (AIDS, leprosy, hepatitis etc.) autoimmune, neuropsychiatric and many other diseases with different SNPs can be made as relevant pharmacogenomic targets for drug therapy (Fareed & Afzal, 2013).

Cytokines are cell-signaling molecules released by cells, which can stimulate the movement of immune modulatory cells towards the sites of infection and inflammation and plays central role in multiple inflammatory responses. It also plays the crucial role of coordination between cell-mediated and humoral immune responses. The outcome of inflammation is characterized by the complex interaction and balance between pro- and anti-inflammatory cytokines (Tayal & Kalra, 2008).

In addition, the genetic variations in cytokine genes are known to modulate their differential expression and hence the balance between pro-inflammatory and anti inflammatory immune response (Ollier, 2004).

- **Tumor Necrosis Factor- α**

Tumor necrosis factor (*TNF*) is a cytokine with pleomorphic actions. *TNF*-α is critical in host defense against infections and has a major role in autoimmune diseases as well. It is also a crucial cytokine for granuloma formation. The level of *TNF*-α varies from among individuals and is genetically determined (Wilson *et al.*, 1992). The gene for *TNF*-α is located within the major histocompatibility complex (MHC) region on chromosome 6p21.3 which is a highly polymorphic region. There are many biallelic single nucleotide polymorphisms (SNPs) in and around the *TNF-α* gene. One such G/A polymorphism is located upstream of gene at -308 and is known to influence *TNF*-α levels (Braun *et al.*, 1996).

TNF-α - 308 promoter gene polymorphism has been reported to be associated with several autoimmune disorders including systemic lupus erythematosus, rheumatoid arthritis and infections such as tuberculosis (Hajeer & Hutchinson, 2000).

El Sissy *et al.* (2014) revealed that the frequency of TNF-α -308A/A homotype in ITP patients was significantly higher than that of the controls, and conferred almost six-fold increased risk of ITP acquisition.

➢ Tumor Necrosis Factor-β

TNF-β is a Th1 cytokine that is produced predominantly by mitogen-stimulated T-lymphocytes and leukocytes. The factor is secreted also by fibroblasts, astrocytes, myeloma cells, endothelial cells, epithelial cells and a number of transformed cell lines. The synthesis of *TNF- β* is stimulated by interferons and IL2. Some pre-B-cell lines and Abelson murine leukemia virus-transformed pre-B-cell lines constitutively produce *TNF-β*. Warzocha *et al.* (1998) suggested that overproduction of *TNF-β* has been implicated in autoimmune disorders and lymphoma.

TNF- β (+252) G/G phenotype was marginally more frequent in adult patients with chronic ITP than in healthy controls in Japanese population. The SNP at *TNF- β* (+252) has been reported to be associated with various autoimmune diseases, including the G allele with systemic lupus erythematosus (Tomita *et al.*, 1992; Bettinotti *et al.*, 1993), the G/A phenotype with Graves' disease (Badenhoop *et al.*, 1992) and the A/A phenotype with systemic sclerosis. Taken together, it is likely that the SNP at *TNF- β* (+252) is a genetic factor that influences the onset of several distinct autoimmune diseases. Foster *et al.* (2001) reported that *TNF- β* (+252) A/A phenotype was higher in Caucasian patients with chronic childhood ITP than in healthy controls.

➢ Interleukin -4

Interleukin 4 (*IL*-4) is an anti-inflammatory cytokine produced by CD4+ Th2 cells, basophils and mast cells. It regulates balance between TH1 and TH2 immune response, induces immunoglobulin class switching and humoral immunity. It promotes TH2 cell differentiation while inhibiting the TH1 cell differentiation and plays a dominant role in immunopathology of diseases (Banchereau *et al.*, 1994; Guo *et al.*, 2002; Murphy & Reiner, 2002; Wurtz *et al.*, 2004)

IL-4 has been observed to exert effects relating to the growth and proliferation of B cells and to the production of serum IgG. The *IL-4* gene is located on the long arm of chromosome 5 (5q23.3-31.2). The major polymorphisms of the *IL-4* gene appear at the promoter -590, a T/C polymorphism, and at intron 3 revealing a 70-bp VNTR. The *IL-4* gene promoter -590 polymorphism has been observed to be associated with asthma and atopic dermatitis, whereas *IL-4* gene polymorphism at intron 3 has been reported to be associated with rheumatoid arthritis and immune thrombocytopenic purpure.

- **Interleukin -10**

IL-10 is a cytokine, which is expressed by cells of the innate and the adaptive immune system, including dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, eosinophils, neutrophils, CD4 & CD8 T cells and B cells. *IL*-10 has potent anti-inflammatory properties, repressing the expression of cytokines such as *TNF*-α, IL-6 and IL-1. The balance between *TNF*- α and IL-10 is important for the maintenance of immune homeostasis (Shmarina *et al.*, 2001). It has been reported that low levels of *IL*-10 are governed by its gene promoter polymorphisms (Abanmi *et al.*, 2008). The *IL10* gene is located on the chromosome 1 and *IL*-10 production appears to be genetically encoded, which has been reported to cause 75% of the variation in IL-10 levels. The highly polymorphic promoter region of human *IL*-10 gene has been found to be associated with numerous autoimmune diseases (Asadullah *et al.*, 2003). Hence, it becomes relevant to investigate *IL10* promoter polymorphisms in Immune thrombocytopenia since it has autoimmune origin.

The objective of this study, therefore, was to examine the genetic association of *TNF* α (-308G/A), *TNF* β (+252A/G), *IL*4 (-590C/T), *IL*4 intron 3 VNTR & *IL*10 (-592C/A; -1082G/A) gene single nucleotide polymorphisms in patients, with Immune Thrombocytopenia Purpura (ITP), from Western belt of India. These cytokines were selected based on the presence of well defined SNPs association with autoimmune diseases.

5.2. Materials and methods:

5.2.1. Experimental Design:

201 subjects regardless of age and sex from Western India were recruited into the study, which included 51 individuals with persistent Immune thrombocytopenia and 150 healthy control individuals. Blood samples of confirmed ITP patients were collected from established oncology clinic. 2-5 ml of peripheral blood was collected in EDTA vacutainers.

5.2.2. DNA isolation and Gene Amplification:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated tubes (BD Vacutainer®, Becton, Dickinson and Company, New Jersey USA). Genomic DNA was extracted from whole blood using ‘whole blood DNA extraction kit’ (HiPurA™ Blood Genomic DNA Miniprep Purification Kit - HiMedia) 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^{o}C until further analyses.

5.2.3 PCR Amplification:

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to genotype *TNFα* (-238G/A), *TNF* β (+252A/G), *IL*4 (-590C/T), *IL*10 (-592C/A; -1082G/A) polymorphisms, whereas PCR was used to genotype *IL*4 intron 3 VNTR.

PCR comprised of 20 µl reaction mixture, which included 50 ng - 150 ng of DNA, 2 µl of 10x Mastermix (Dream Taq Mastermix, Thermo Scientific, USA) and 0.5 µM of each primer. For amplification, an initial denaturation step at 95°C for 3 min. followed by 35 cycles at 95°C for 30sec. at Primer denaturation temperature and at 72°C for 30 sec. followed by final extension for 10 min at 72°C was carried out. Subsequently for each SNP the PCR product (amplicon) was digested with respective Restriction enzymes. The respective Restriction enzymes and digestion condition are mentioned in Table 5.1. Finally digested products were visualized on 3.5 % agarose gel and further analyzed on Biorad Gel Doc™ EZ System (BIORAD, USA).

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for polymorphisms in both the 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 polymorphisms for patients and control subjects were compared using the chi-squared test with 3x2 and 2x2 contingency tables respectively using Prism 6 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility were also calculated.

5.2.4. Primer Sequences

Table 5.1: Primer Sequences

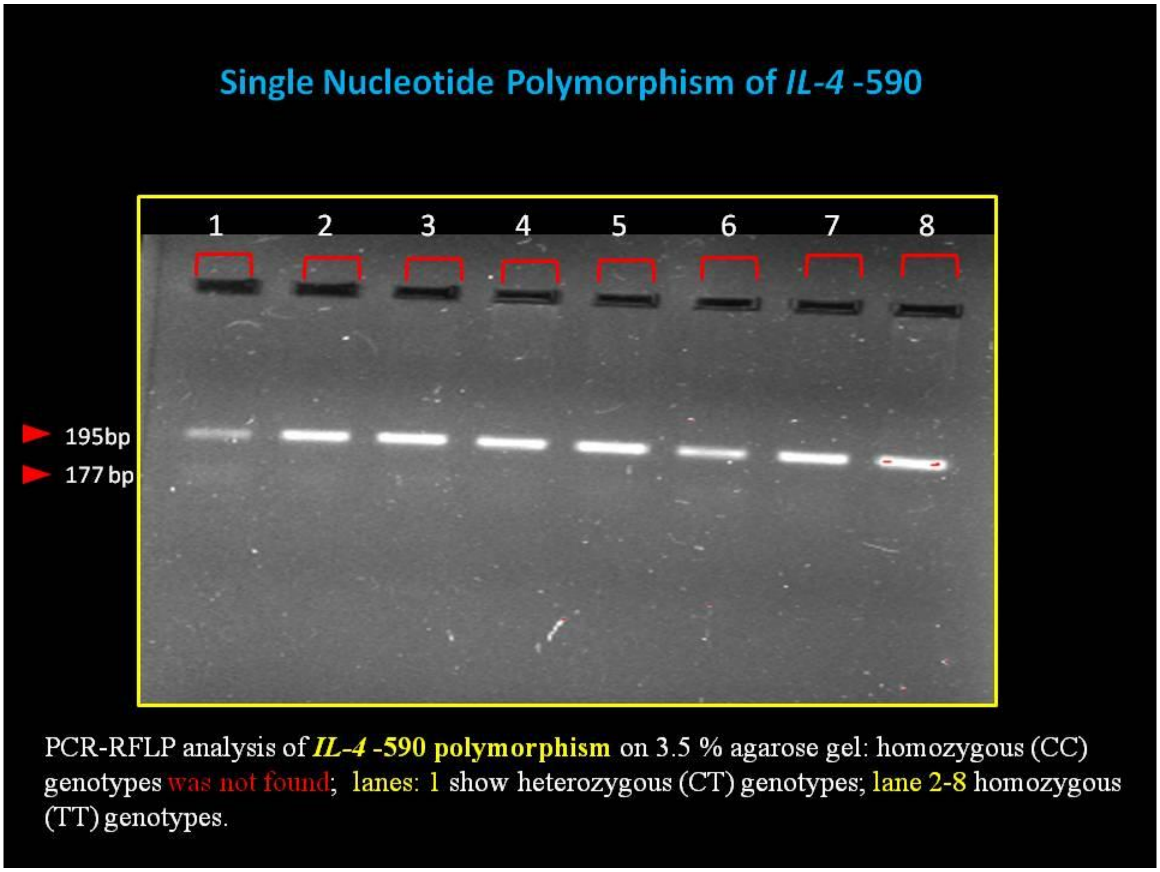
Gene/SNP	**Primer Sequence (5' to 3')**	**Amplicon size (bp)**	**Annealing Temperature (°C)**	**Digested Products**
TNF α -308	FP: GAG GCA ATA GGT TTT GAG GGC CAT	380 bp	60°C	290
	RP: TCT GCT GTC CTT GCT GAG GGA			90
TNF β +252	FP: GGTGGTGTCATGGGGAGAACC	315	62°C	218
	RP: GGGCCTTGGTGGGTTTGGTT			97
IL-4 -590	FP: TAAACTTGGGAGAACATGGT	195 bp	51°C	177
	RP: TGGGGAAAGATAGAGTAATA			18
IL-4 Int 3 VNTR	FP: AGGCTGAAAGGGGGAAAGC	253 /183 bp	60°C	NA
	RP: CTGTTCACCTCAACTGCTCC			NA
IL-10 -592	FP: TGGTGAGCACTACCTGACTAGC	413 bp	58°C	236
	RP: CCTAGGTCACAGTGACGTGGAC			177
IL-10 -1082	FP: CTCGCCGCAA CCCAACTGGC	180 bp	64°C	151
	RP: GGTCCCTTACTTTCGTCTTACCTATCC			30

5.2. Restriction Enzymes details

TABLE 5.2: Respective restriction enzymes used in PCR – RFLP and their properties

SNP Type	Restriction Enzyme	Restriction Sites	Digestion Conditions Activation/Inactivation
***TNF α* (-308G/A)**	NCOI	…C▼CGG… …GGC▲C…	37°C / 4 °C
***TNFβ* (+252A/G)**	Nco I	…C▼CATGG… …GGTAC▲C…	37°C/ 80 °C
***IL*4 (-590C/T)**	Ava II	…G▼GWCC… …CCWG▲G…	37°C / 80 °C
***IL*10 (-592C/A)**	Rsa I	…G▼TAC… …CAT▲G…	37°C / 80 °C
***IL*10 (-1082G/A)**	Mnl I	…CCTC $(N)_7$▼… ...GGAG $(N)_6$.▲.	37°C / 65 °C

5.3. RESULTS

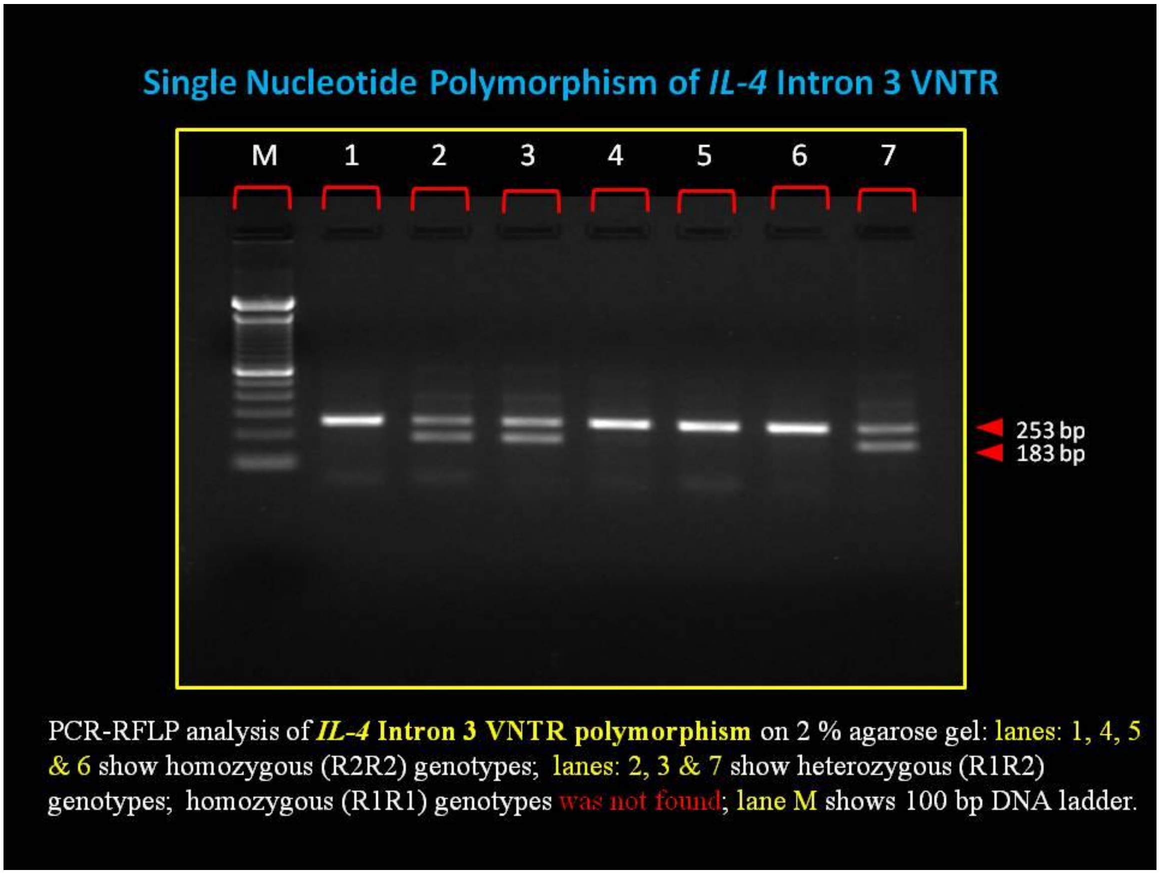


PCR-RFLP analysis of ***IL-4* -590 polymorphism** on 3.5 % agarose gel: homozygous (CC) genotypes was not found; lanes: 1 show heterozygous (CT) genotypes; lane 2-8 homozygous (TT) genotypes.

Figure 5.1: Representative PCR-RFLP gel image of *IL*-4-590 polymorphism

SNP	**Genotype/ Allele**	**Controls (n=103)**	**Patients (n=42)**	**p value# for Association**	**OR**	**95% CI**
***IL4* C-590T**	CC	57 (0.55)	1 (0.02)	R	1	
	CT	33 (0.32)	5 (0.12)	0.0006	10.31	2.168-49.05
	TT	13 (0.13)	36 (0.86)	<0.0001	194.8	41.96-904.3
	C	147 (0.71)	7 (0.08)	R	1	-
	T	59 (0.29)	77 (0.92)	<0.0001	28.16	12.13-65.34

Table 5.3: Distribution of *IL*-4 C-590T genotype in ITP patients



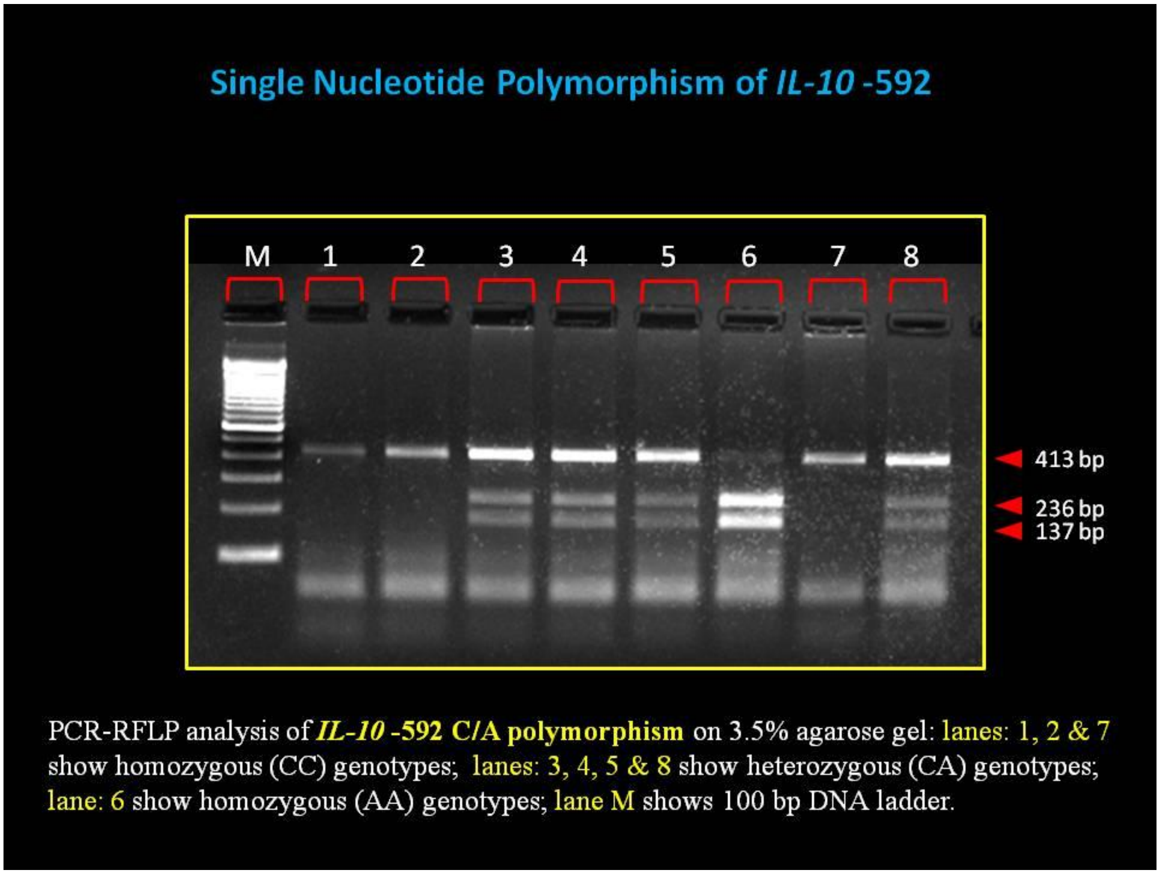
Single Nucleotide Polymorphism of *IL-4* Intron 3 VNTR

PCR-RFLP analysis of ***IL-4*** **Intron 3 VNTR polymorphism** on 2 % agarose gel: lanes: 1, 4, 5 & 6 show homozygous (R2R2) genotypes; lanes: 2, 3 & 7 show heterozygous (R1R2) genotypes; homozygous (R1R1) genotypes was not found; lane M shows 100 bp DNA ladder.

Figure 5.2: Representative PCR-RFLP gel image of *IL*-4-Intron 3 VNTR polymorphism

SNP	**Genotype/ Allele**	**Controls (n=103)**	**Patients (n=52)**	**p value# for Association**	**OR**	**95% CI**
VNTR *IL-4* Intron 3	R1R1	14 (0.13)	0 (00)	R	1	
	R1R2	68 (0.66)	17 (0.33)	0.0102	14.61	0.8448 to 252.6
	R2R2	21 (0.20)	35 (0.67)	< 0.0001	95.49	5.453 to 1672
	R2	110 (0.53)	87 (0.84)	R	1	-
	R1	96 (0.47)	17 (0.16)	< 0.0001	4.656	2.3982-9.039

Table 5.4: Distribution of *IL*-4-Intron 3 VNTR genotype in ITP patients


Single Nucleotide Polymorphism of *IL-10* -592

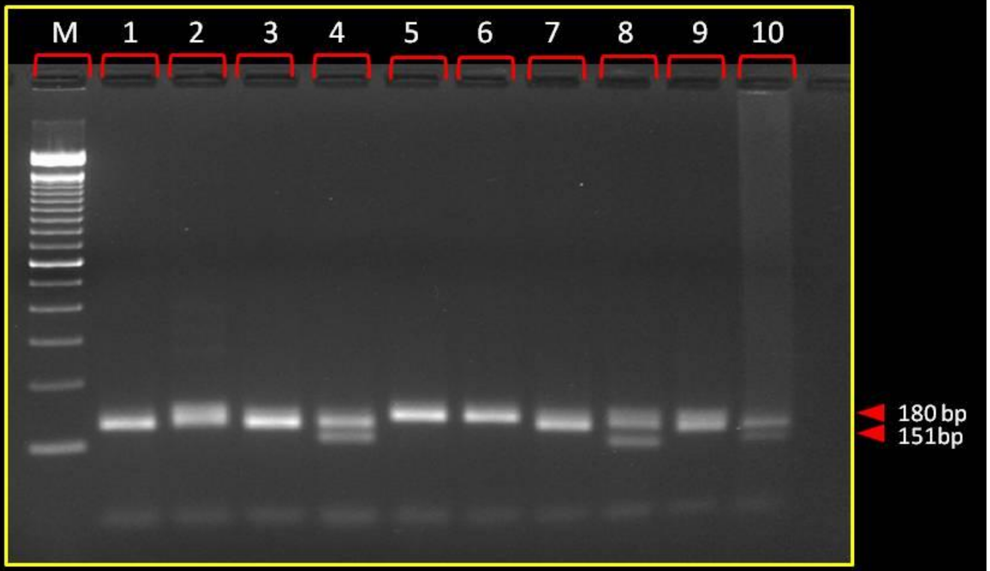
PCR-RFLP analysis of ***IL-10* -592 C/A polymorphism** on 3.5% agarose gel: lanes: 1, 2 & 7 show homozygous (CC) genotypes; lanes: 3, 4, 5 & 8 show heterozygous (CA) genotypes; lane: 6 show homozygous (AA) genotypes; lane M shows 100 bp DNA ladder.

Figure 5.3: Representative PCR-RFLP gel image of IL-10-592 polymorphism

SNP	**Genotype/ Allele**	**Controls (n=103)**	**Patients (n=48)**	**p value# for Association**	**OR**	**95% CI**
***IL10* - C592T**	CC	34 (0.33)	20 (0.42)	R		
	CA	56 (0.54)	19 (0.39)	0.0941	0.5930	0.3210-1.095
	AA	13 (0.13)	09 (0.19)	0.7468	1.148	0.4957-2.660
	C	124 (0.6)	59 (0.61)	R	1	-
	A	82 (0.4)	37 (0.39)	0.8850	0.9590	0.5439-1.691

Table 5.5: Distribution of **IL-10-592 genotype in ITP patients**

Single Nucleotide Polymorphism of *IL-10* -1082

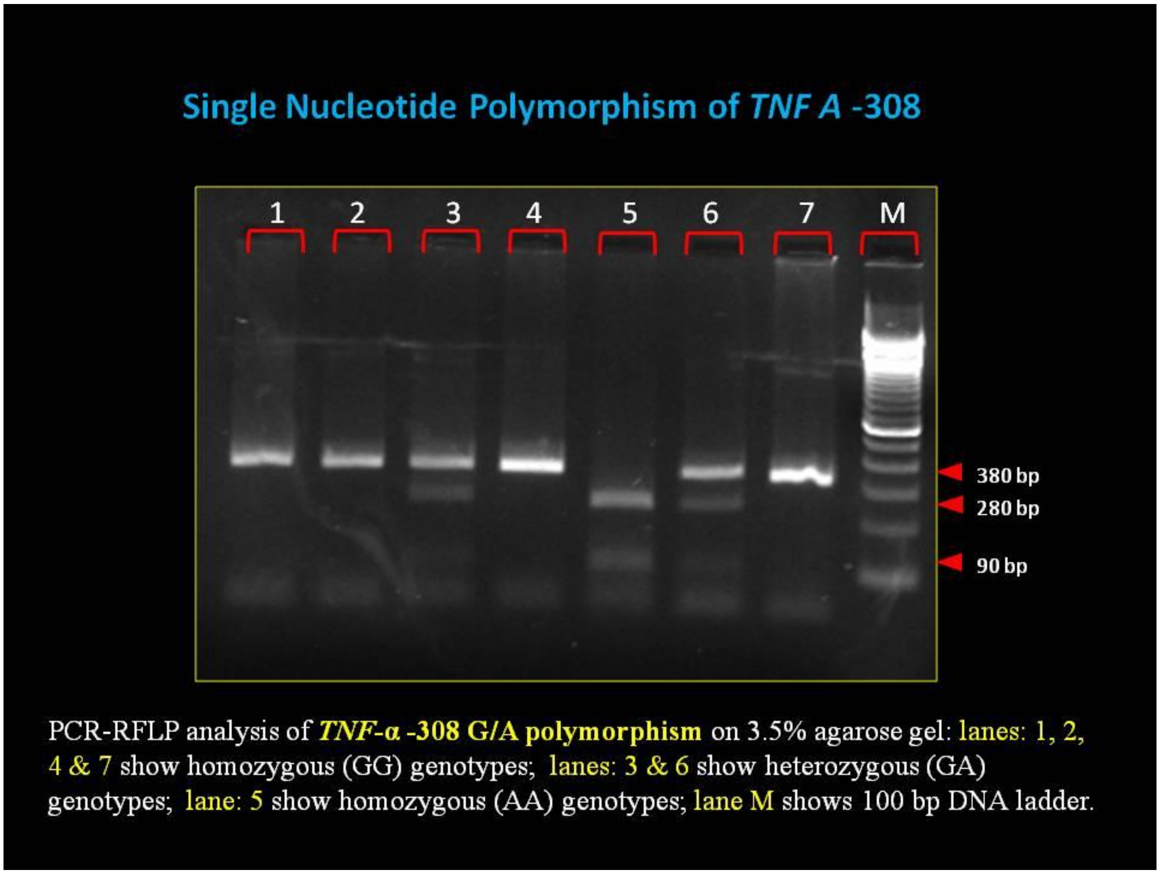


PCR-RFLP analysis of ***IL-10* -1082 G/A polymorphism** on 3.5% agarose gel: lanes: 2, 5 & 6 show homozygous (GG) genotypes; lanes: 4, 8 & 10 show heterozygous (GA) genotypes; lane: 1,3, 7 &9show homozygous (AA) genotypes; lane M shows 100 bp DNA ladder.

Figure 5.4: Representative PCR-RFLP gel image of *IL*-10-1082 polymorphism

SNP	**Genotype / Allele**	**Controls (n=103)**	**Patients (n=51)**	**p value# for Association**	**OR**	**95% CI**
***IL10* – *G*-1082A**	GG	48 (0.47)	13 (0.25)	R	1	
	GA	30 (0.29)	17 (0.33)	0.398*	2.057	1.030-4.109
	AA	25 (0.24)	21 (0.41)	0.0013**	3.088	1.541-6.190
	G	126 (0.61)	43 (0.42)	R	1	-
	A	80 (0.39)	59 (0.58)	0.0072	2.160	1.228-3.801

Table 5.6: Distribution of *IL*-10-1082 genotype in ITP patients

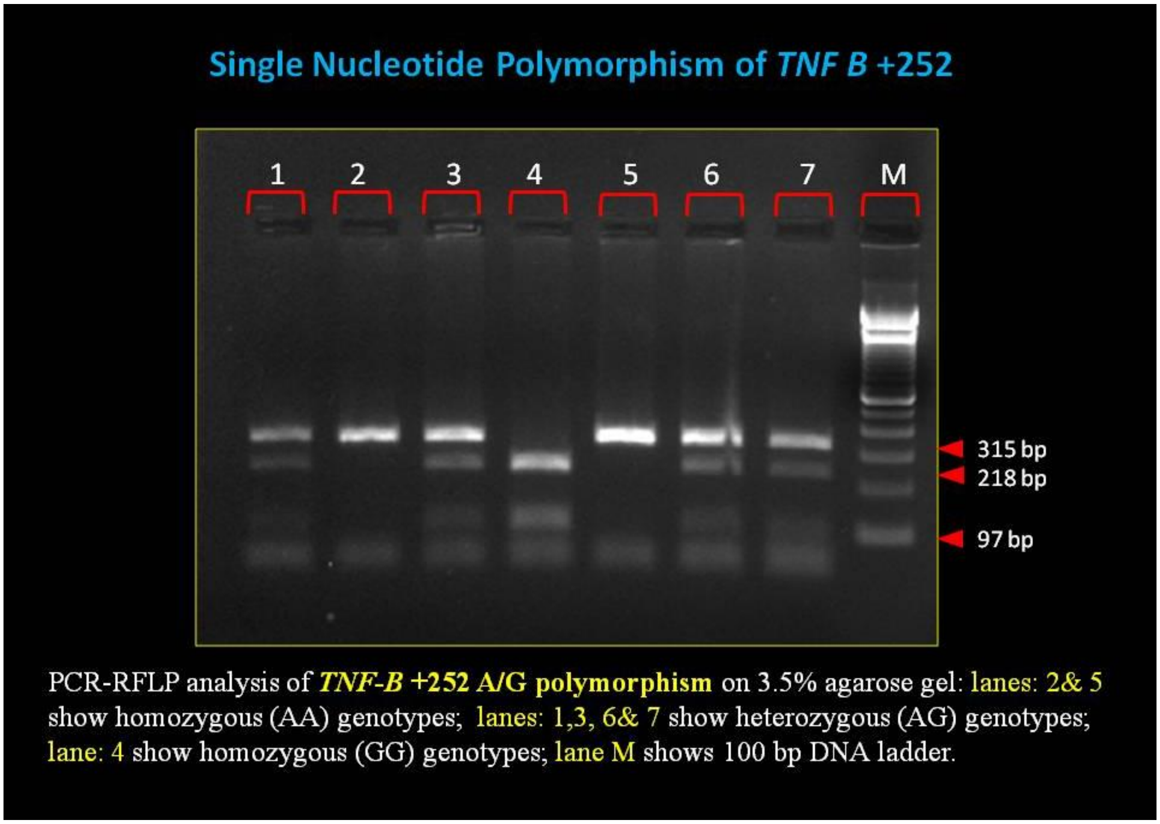


PCR-RFLP analysis of ***TNF*-α -308 G/A polymorphism** on 3.5% agarose gel: lanes: 1, 2, 4 & 7 show homozygous (GG) genotypes; lanes: 3 & 6 show heterozygous (GA) genotypes; lane: 5 show homozygous (AA) genotypes; lane M shows 100 bp DNA ladder.

Figure 5.5: Representative PCR-RFLP gel image of *TNF*-α-308 G/A polymorphism

SNP	Genotype/ Allele	Controls (n=103)	Patients (n=50)	p value# for Association	OR	95% CI
***TNF α* G-308A**	GG	85 (0.83)	13 (0.26)	R		
	GA	18 (0.17)	21 (0.42)	<0.0001	7.887	3.857-16.13
	AA	0 (0.00)	16 (0.32)	<0.0001	204.8	12.11-3463
	G	188 (0.91)	47 (0.47)	R	1	-
	A	18 (0.09)	53 (0.53)	<0.0001	11.40	5.176-25.11

Table 5.7: Distribution of TNFA G-308A genotype in ITP patients



PCR-RFLP analysis of ***TNF-B* +252 A/G polymorphism** on 3.5% agarose gel: lanes: 2& 5 show homozygous (AA) genotypes; lanes: 1,3, 6& 7 show heterozygous (AG) genotypes; lane: 4 show homozygous (GG) genotypes; lane M shows 100 bp DNA ladder.

Figure 5.6: Representative PCR-RFLP gel image of *TNF-* β +252 A/G polymorphism

SNP	Genotype/ Allele	Controls (n=103)	Patients (n=52)	p value# for Association	OR	95% CI
***TNFβ* G+252A**	AA	65 (0.63)	32 (0.62)	R	1	
	AG	33 (032)	18 (0.35)	0.7274	1.111	0.6136-2.013
	GG	5 (0.05)	2 (0.04)	0.765	0.812	0.2084-3.171
	A	163 (0.79)	82 (0.79)	R	1	-
	G	43 (0.21)	22 (0.21)	1	1	0.5063-1.975

Table 5.8: Distribution of *TNF* β G+252A genotype in ITP patients

Discussion:

ITP is an acquired autoimmune disorder that is the most common cause of isolated thrombocytopenia in children. ITP results from the production of antiplatelet autoantibodies in blood. These autoantibodies opsonize platelets for splenic clearance, resulting in thrombocytopenia. The disease may be mediated by autoreactive B lymphocytes, which produce antiplatelet antibodies. Also, increased activation of T cells and cytokine response suggest that T-lymphocytes could play an important role in this autoimmune process. Genetic factors such as gene polymorphisms, including those in cytokine genes, have been reported to be associated with ITP. It is known that Th1 predominance can induce autoimmunity, whereas Th2 predominance can inhibit the immune response. The function of Th1 cells is to promote cellular immunity by secreting interferon (IFN)-γ, interleukin (*IL*) 2 and tumor necrosis factor (*TNF*)-α. In contrast, Th2 cells mostly induce humoral immunity by producing *IL*4, *IL*5, *IL*6, *IL*10, and *IL*13. Allelic variations in regulatory regions of cytokine genes have shown to be responsible for the altered expression of some cytokines. ITP has been associated with dysregulation of the cytokine response.

It has been proposed that chronic ITP is characterized by a decrease in Th2 cell counts. A high Th1/Th2 ratio was closely related to the etiology and disease status of chronic ITP. In ITP, contact between macrophages, dendritic cells, and foreign antigens may initiate a proinflammatory cytokine cascade characterized by *IL*1, *TNF*α, *IL*6, and *IL*8 production followed by a chemokine burst and a counter-response of antiinflammatory cytokines such as *IL*1Ra, tumor growth factor-β, and *IL*10. The *IL4* gene has a 70-bp VNTR polymorphism in intron 3 associated with *IL*4 production (Wu *et al.*, 2005). *IL*4 intron 3

VNTR polymorphisms account for the overproduction of this cytokine, which in turn affects the magnitude and duration of the immune response, perhaps predisposing the individual to autoimmune disorders.

In our study, genotyping detected *IL*4 VNTR intron 3, R1/R1, R1/R2, and R2/R2 in 00%, 33%, and 67% of ITP patients, respectively. Our results were distinctly contrast from earlier reports by Li *et al.* (2014) in Chinese patients R1/R1, R1/R2, R2/R2 (62.7%, 34.7% and 8.0% respectively), and Makhlouf and Elhamid (2014) R1/R1, R1/R2, R2/R2 (55%, 35% and 10% respectively)

Although the function of the intron 3 polymorphism of the *IL*4 gene is not known, it is possible that distinct numbers of VNTR might affect the transcriptional activity of the *IL*4 gene. Some studies have provided evidence suggesting that the R1 allele induces higher expression of IL4 than the R2 allele, and that the R1 allele may be a protective factor in some diseases. The frequency of R1 allele among the patient population in our study was significantly lower as compared to the control population suggesting its association with ITP (Table 5.4)

IL-4 is the Th2 cytokine that is pivotal for the pathogenesis of many autoimmune diseases; it induces the differentiation of Th0 cells to Th2 cells (Gadani *et al.*, 2012). Th2 cells subsequently produce additional IL-4 in a positive feedback mechanism upon activation by IL-4 (Gadani *et al.*, 2012). There are some *IL-4* polymorphisms which affect the expression level of IL-4, including *IL-4* VNTR intron 3 and *IL-4*-590C/T. Rosenwasser *et al.* (1995) analyzed the association between IL-4 production and the *IL-4* -590C/T polymorphism, and reported the TT genotype was linked to higher IL-4 levels compared to the C/C genotype. Several *IL-4* -590C/T polymorphism studies were reported in various autoimmune diseases, including asthma, rheumatoid arthritis, and multiple sclerosis (Arababadi *et al.*, 2012; Berenguer *et al.*, 2014; Li *et al.*, 2014). We found significant association between *IL-4* -590C/T polymorphism and susceptibility to cITP. The TT allele was significantly higher in chronic ITP patients as compared to the control group (86% v/s 13%) (Table 5.3).

In our chronic ITP patients, IL10 C/C, C/A, and A/A genotypes were detected in 42%, 39%, and 19% respectively. These results slightly differed from that reported in Chinese patients by Wu *et al.* (2003) (60.0%, 13.3%, and 26.7% respectively).

The frequency of IL10 (-592) C/C genotype was higher in ITP patients compared to controls (42% versus 33%). Wu *et al.* (2003) stated that the wild type C/C was statistically higher in patients than control (52.5% versus 41.0%) which was in agreement with our findings (Wu *et al.*, 2005). The IL-1082 showed significant association with chronic ITP patients from Gujarat population. Similar results were also reported by Mokhtar *et al.* (2016) who reported higher GG of IL10 -1082 in childhood ITP patients. They also found best platelet response to steroid treatment among IL10 -1082 in all patients (Table 5.5, 5.6).

We have examined potential associations between the development of chronic ITP and the SNPs within the genes for several inflammatory cytokines, and found that the *TNF-β* (+252) A/G phenotype was not found altered in adult patients with chronic ITP when compared with healthy controls (AA 61% , AG 35% , GG 04%) v/s (AA 63% , AG 32% , GG 05%). The SNP at *TNF-β* (+252) has been reported to be associated with various autoimmune diseases, including the G allele with systemic lupus erythematosus (Tomita *et al.,* 1992; Bettinotti *et al.*, 1993), the G/A phenotype with Graves' disease (Badenhoop *et al.*, 1992) and the A/A phenotype with systemic sclerosis (Pandey & Takeuchi, 1999) ,whereas a lack of significant association was reported for patients with Myasthenia Gravis (Zelano *et al.*, 1998), multiple sclerosis, rheumatoid arthritis (Vandeyver *et al.*, 1994) and Behcet's disease (Lee *et al.*, 2003). Studies carried out by Foster *et al.* (2001) has reported that the *TNF-β* (+252) A/A phenotype was higher in Caucasian patients with childhood chronic ITP than in healthy controls.

In our studies we found *TNF* α (-308) (AA & AG) significantly associated with chronic ITP. The genotypes GG 26%, GA 42% and AA 32% showed marked difference when compared with the genotype GG 83%, GA 17% and AA 00% of control population. Several studies have reported that polymorphism in *TNF*-α 308G>A contributes to susceptibility to ITP. Mokhtar *et al.* (2016) reported higher GA genotype of *TNF* α (-308) (p=0.001), which is in agreement with our results (Table 5.7). Zhang *et al.* (2017) suggested that *TNF*-α -308G/A might be involved in development of ITP in the Caucasian population, but not in the Asian population and among Caucasians the A allele (AA+AG) was associated with ITP. This might be explained by the fact that *TNF-α* is a pro-inflammatory cytokine involved in different immune-regulated diseases including autoimmune, infectious, and malignant ones (Lee *et al.,* 2006).

To summarize, in our attempt to associate cytokine gene polymorphism, we found among the six genes studied four (IL4 VNTR, *IL* 4, *IL*10 -1082) genes showed significant association while *IL*10 -592 and *TNF* β did not show any association in the chronic ITP patients under study from Gujarat, India (Table 5.8). These results were in agreement with some researchers while they differed from the others. Notably, the results of Yadav *et al.* (2016) showed no association of *TNF*- α with susceptibility in developing ITP but significant association of heterozygous variant (AG) genotype of *TNF*-β in primary ITP patients from North India.

The reason for the difference between the earlier reports and our finding is unclear, but possible explanations include differences in the patients' age, ethnic background the geographic distribution or the different sample sizes used in these studies. Also the data on association of cytokine gene polymorphism with ITP are currently meager for Indian population. Therefore, taking into consideration the limiting factors, we recommend undertaking a wide-scale multicenter study into the genome sequence studying all cytokine gene polymorphisms and their expression status in cases of ITP throughout Indian population.

5.5. BIBLIOGRPAHY

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