CHAPTER VII

ROLE OF TUMOR NECROSIS FACTOR β (TNFβ) IN VITILIGO SUSCEPTIBILITY

7.1 INTRODUCTION

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). 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.

Tumour necrosis factor (TNF)-β also known as lymphotoxin α (LTα), is a close homologue of TNFα. TNFβ has close structural homology and about 30% amino acid sequence identity to TNFα. Both of these cytokines are recognized by the same widely distributed cellular TNF receptors (Smith, 1994) and as a consequence, many of their numerous effects are similar. TNFβ is a Th1 cytokine, primarily produced by activated T and B lymphocytes. TNFβ is a potent mediator of inflammatory and immune responses and it is also involved in the regulation of various biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, coagulation, and neurotransmission.

The *TNFB* gene is of 3 kb and contains four exons. It encodes a primary transcript of 2038 nucleotides yielding a mRNA of 1.4 kb. The genes for the cytokines *TNFA* and *TNFB* are tandemly arranged in the class III region of the 3.6 Mb major histocompatibility complex (MHC) between the *HLA*-B and *HLA*-D on chromosome 6p21.3-21.1. *TNFB* is present approximately 1.2 kb apart from the *TNFA* gene. However, both genes are regulated independently. The 5' region of the *TNFB* promoter contains a poly(dA-dT)-rich sequence that binds the nonhistone protein HMG-1 which is involved in its constitutive expression. The synthesis of TNFβ is stimulated by interferons and IL-2. TNFβ is a protein of 171 amino acids with N-glycosylation at position 62. TNFβ is secreted as a soluble polypeptide, but can form heterotrimers with lymphotoxin-beta, which effectively anchors the TNFβ to the cell surface. The soluble TNFβ can interact with TNF receptors 1 (TNFR1) and 2 (TNFR2), similar to TNFα (Funahashi *et al.*, 1991). TNFβ induces cell apoptosis upon binding to TNF receptor type 1, but it induces inflammatory responses by activating NF-kB nuclear protein upon binding to TNF receptor type 2 (Lucas *et al.*,

1997). TNFβ is involved in a large variety of inflammatory, immune-stimulatory, and antiviral responses (Vassalli, 1992).

Polymorphisms of the proinflammatory and immune-regulatory cytokines, *TNFA* and *TNFB* genes have been shown to affect their production and hence can be associated with several autoimmune diseases (Vassalli, 1992; Beutler and Bazzoni, 1998). It is possible that individuals who naturally produce higher levels of these cytokines might exhibit different susceptibility, or severity towards autoimmune diseases.

Two well-characterized variants of *TNFB* are in tight linkage disequilibrium; +252A/G polymorphism in the first intron (IVS1+90 A/G) and (Thr26Asn) causing the substitution of a threonine residue with an asparagine residue at codon 60 in exon 3 (Messer *et al.*, 1991) which was found to influence *in vitro TNFB* expression (Messer *et al.*, 1991; Whichelow *et al.*, 1996). The +252A/G polymorphism also reported to influence TNFβ plasma levels. This single nucleotide polymorphism (C252 A/G) affects a phorbol ester-response element and distinguishes the two alleles that have been designated as *TNFB1* (5.5 kb) and *TNFB2* (10.5 kb) alleles (Messer *et al.*, 1991). *TNFA2* and *TNFB2* alleles are much more powerful transcription activators than *TNFA1* and *TNFB1* alleles, respectively (Messer *et al.*, 1991). Other *in vitro* experiments have shown that the Thr26Asn polymorphism is associated with a twofold increase in the induction of several cell-adhesion molecules including VCAM1 and ICAM1 in the smooth muscle cells of human coronary arteries (Ozaki *et al.*, 2002).

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

i.). To determine whether the promoter polymorphisms of *TNFB* [intron 1 +252G/A (rs909253) and exon 3 A/C (rs1041981; Thr26Asn)] are associated with vitiligo susceptibility and modulate *TNFB* transcript levels.

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

iii.)To correlate *TNFB* polymorphisms and its transcript levels with the onset and progression of disease.

7.2 MATERIALS AND METHODS

7.2.1 Study Subjects:

The study group included 524 vitiligo patients [360 generalized (including acrofacial vitiligo and vitiligo universalis) and 164 localized vitiligo cases] comprised of 224 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 592 ethnically sex-matched unaffected individuals (267 males and 325 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 the patients and controls.

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

	Vitiligo Patients	**Controls**
	(n = 524)	(n = 592)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	224 (42.75%)	267 (45.10%)
Female	300 (57.25%)	325 (54.90%)
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	360 (68.70%)	NA
Localized	164 (31.30%)	NA
Active vitiligo	393 (75.00%)	NA
Stable vitiligo	131 (25.00%)	NA
Family history	68	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 *TNFB* +252G/A polymorphism:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype +252G/A polymorphisms of *TNFB* 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 35 cycles of 95°C for 30 seconds, 62°C 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 enzyme *Nco*I (New England Biolabs, Beverly, MA) was used for digesting amplicons of +252G/A of *TNFB* gene (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 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).

Table 2. Primers and restriction enzymes used for *TNFB* +252G/A SNP genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs909253)				
TNFB +252G/A F	GGTGGTGTCATGGGGAGAACC	62	417	*Nco*I (284 & 137 bp)
TNFB +252G/A R	GGGCCTTGGTGGGTTTGGTT			
TNFB expression F	GGGCCTTGGTTCTCCCCATG	65	232	-
TNFB expressionR	CTGGGGTCTCCAATGAGGTGA			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expressionR	CAAATGAGCCCCAGCCTTCT			

7.2.4 Genotyping of *TNFB* Thr26Asn A/C polymorphism:

The genotyping of Thr26Asn A/C SNP of *TNFB* was carried out by dual color hydrolysis probes (FAM and VIC) using LightCycler® 480Real-Time PCR protocol with background corrected end point fluoroscence analysis using TaqMan SNP genotyping assay (Assay ID: C_7514870_20; 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 *TNFB* and *GAPDH* mRNA expression:

7.2.5.1 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 RNA purity by confirming 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).

7.2.5.2 Real-time PCR:

The expression of *TNFB* 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 5). 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 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 *TNFB* 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.
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 r2-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 *TNFB* 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 *TNFB* +252G/A polymorphism and susceptibility to vitiligo:

PCR-RFLP for *TNFB* +252G/A polymorphism yielded a 417 bp undigested product corresponding to A allele and 284 bp and 133 bp digested products corresponding to G allele. The three genotypes identified by 2.0% agarose gel electrophoresis were: GG homozygous, GA heterozygous and AA homozygous for +252G/A polymorphism of *TNFB* gene (Figure 1).

The genotype and allele frequencies of the +252G/A polymorphism in 524 vitiligo patients and 592 controls are summarized in Table 3. The *TNFB* +252G/A polymorphism was found to be in significant association with vitiligo patients (p=0.0024) when genotypes were compared using chi-squared test-3x2 contingency table (Table 3). The minor allele (G) of +252G/A was more frequent in the vitiligo

group compared to the control group (27.0% versus 21.0%, p=0.0005; OR 1.424, 95% CI 1.171-1.732) consistent with a susceptibility effect (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.389 and p=0.109 respectively) (Table 3). Moreover, generalized vitiligo group showed significant association of +252G/A polymorphism when the genotypes were compared with those of control group (p<0.0001); however localized vitiligo group did not show significant association of the polymorphism (p=0.826) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized vitiligo group when the alleles were compared with those of control group (p<0.0001); however, localized vitiligo group did not show significant difference in allele frequencies (p=0.759) (Table 4). The distribution of *TNFB* +252G/A genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05). In addition, gender based analysis of *TNFB* +252G/A polymorphism suggested significant association of minor +252G allele with female vitiligo patients as compared to male patients (30.0% versus 23.0%, p=0.021); however, genotype frequencies could not achieve significance due to Bonferroni's correction for multiple testing (p=0.039) (Table 5). This study has 84.96% 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.

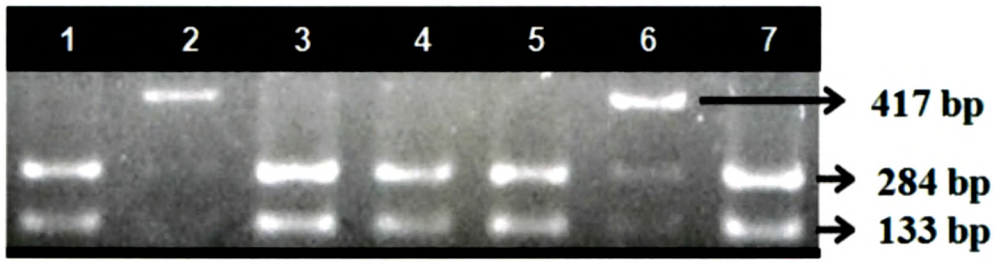


Figure 1. PCR-RFLP analysis of *TNFB* +252G/A polymorphism on 2.0 % agarose gel electrophoresis: lanes: 1, 3, 4, 5 & 7 show homozygous (GG) genotypes; lane: 2 shows homozygous (AA) genotype; lane: 6 shows heterozygous (GA) genotype.

Table 3. Association studies for *TNFB* gene +252G/A and exon 3 A/C 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 = 524)	(n = 592)		0.109	
	GG	46 (0.09)	29 (0.05)		(P)	
rs909253	GA	193 (0.37)	188(0.32)	0.0024[a]		
(+252G/A)	AA	285 0.54)	375(0.63)		0.389	
	Allele				(C)	1.424
	G	285 (0.27)	246(0.21)	0.0005[b]		(1.171-
	A	763 (0.73)	938(0.79)			1.732)
	Genotype	(n = 524)	(n = 592)			
	AA	46 (0.09)	29 (0.05)		0.109	
	AC	193 (0.37)	188(0.32)	0.0024[a]	(P)	
rs1041981	CC	285 (0.54)	375(0.63)			
(Exon 3	Allele				0.389	1.424
A/C;	A	285 (0.27)	246(0.21)	0.0005[b]	(C)	(1.171-
Thr26Asn)	C	763 (0.73)	938(0.79)			1.732)

'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 *TNFB* gene +252G/A and exon 3 A/C 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 = 360)	(n = 164)	(n = 592)			1.623
	GG	39 (0.11)	10 (0.06)	29 (0.05)		0.083	(1.312-
rs909253	GA	137 (0.38)	51 (0.31)	188(0.32)	<0.0001[a]	(GV)	2.008)
(+252G/A)	AA	184 (0.51)	103 (0.63)	375(0.63)	0.826[b]		(GV)
	Allele					0.286	
	G	215 (0.30)	71 (0.22)	246(0.21)	<0.0001[c]	(LV)	1.053
	A	505 (0.70)	257 (0.78)	938(0.79)	0.759[d]		(0.7819
						0.389	-1.419)
						(C)	(LV)
	Genotype	(n = 360)	(n = 164)	(n = 592)			
	AA	39 (0.11)	10 (0.06)	29 (0.05)		0.083	1.623
	AC	137 (0.38)	51 (0.31)	188(0.32)	<0.0001[a]	(GV)	(1.312-
rs1041981	CC	184 (0.51)	103 (0.63)	375(0.63)	0.826[b]		2.008)
(Exon 3	Allele					0.286	(GV)
A/C;	A	215 (0.30)	71 (0.22)	246(0.21)	<0.0001[c]	(LV)	
Thr26Asn)	C	505 (0.70)	257 (0.78)	938(0.79)	0.759[d]		1.053
						0.389	(0.7819
						(C)	-1.419)
							(LV)

'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 *TNFB* gene +252G/A and exon 3 A/C 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 = 224)	(n= 300)			
	GG	12 (0.05)	34 0.11)		0.909	
rs909253	GA	81 (0.37)	112(0.38)	0.039[a]	(M)	
(+252G/A)	AA	131(0.58)	154(0.51)			
	Allele				0.054	0.7143
	G	105(0.23)	180(0.30)	0.021[b]	(F)	(0.5399-
	A	343(0.77)	420(0.70)			0.9449)
	Genotype	(n = 224)	(n = 300)			
	AA	12 (0.05)	34 (0.11)		0.909	
	AC	81 (0.37)	112(0.38)	0.039[a]	(M)	0.7143
rs1041981	CC	131(0.58)	154(0.51)			(0.5399-
(Exon 3	Allele				0.054	0.9449)
A/C;	A	105(0.23)	180(0.30)	0.021[b]	(F)	
Thr26Asn)	C	343(0.77)	420(0.70)			

'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.

7.3.2 Analysis of association between *TNFB* A/C; Thr26Asn 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 'A' allele) and VIC (for 'C' allele) fluorophores for *TNFB* A/C polymorphism which yield the three genotypes (AA homozygous, AC heterozygous and CC homozygous) as identified by scattered plot using background corrected end point fluoroscence analysis (Figure 2).

The genotype and allele frequencies of the *TNFB* A/C polymorphism in 524 vitiligo patients and 592 controls are summarized in Table 3. The *TNFB* A/C polymorphism

was found to be in significant association with vitiligo patients (p=0.0024) when genotypes were compared using chi-squared test-3x2 contingency table (Table 3). The minor allele (A) of *TNFB* A/C was more frequent in the vitiligo group compared to the control group (27.0% versus 21.0%, p=0.0005; OR 1.424, 95% CI 1.171-1.732) consistent with a susceptibility effect (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.389 and p=0.109 respectively) (Table 3). Moreover, generalized vitiligo group showed significant association of *TNFB* A/C polymorphism when the genotypes were compared with those of control group (p<0.0001); however localized vitiligo group did not show significant association of the polymorphism (p=0.826) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized vitiligo group when the alleles were compared with those of control group (p<0.0001); however, localized vitiligo group did not show significant difference in allele frequencies (p=0.759) (Table 4). The distribution of *TNFB* A/C genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05). In addition, gender based analysis of *TNFB* A/C polymorphism suggested significant association of minor (A) allele with female vitiligo patients as compared to male patients (30.0% versus 23.0%, p=0.021); however, genotype frequencies could not achieve significance due to Bonferroni's correction for multiple testing (p=0.039) (Table 5). This study has 84.96% 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.

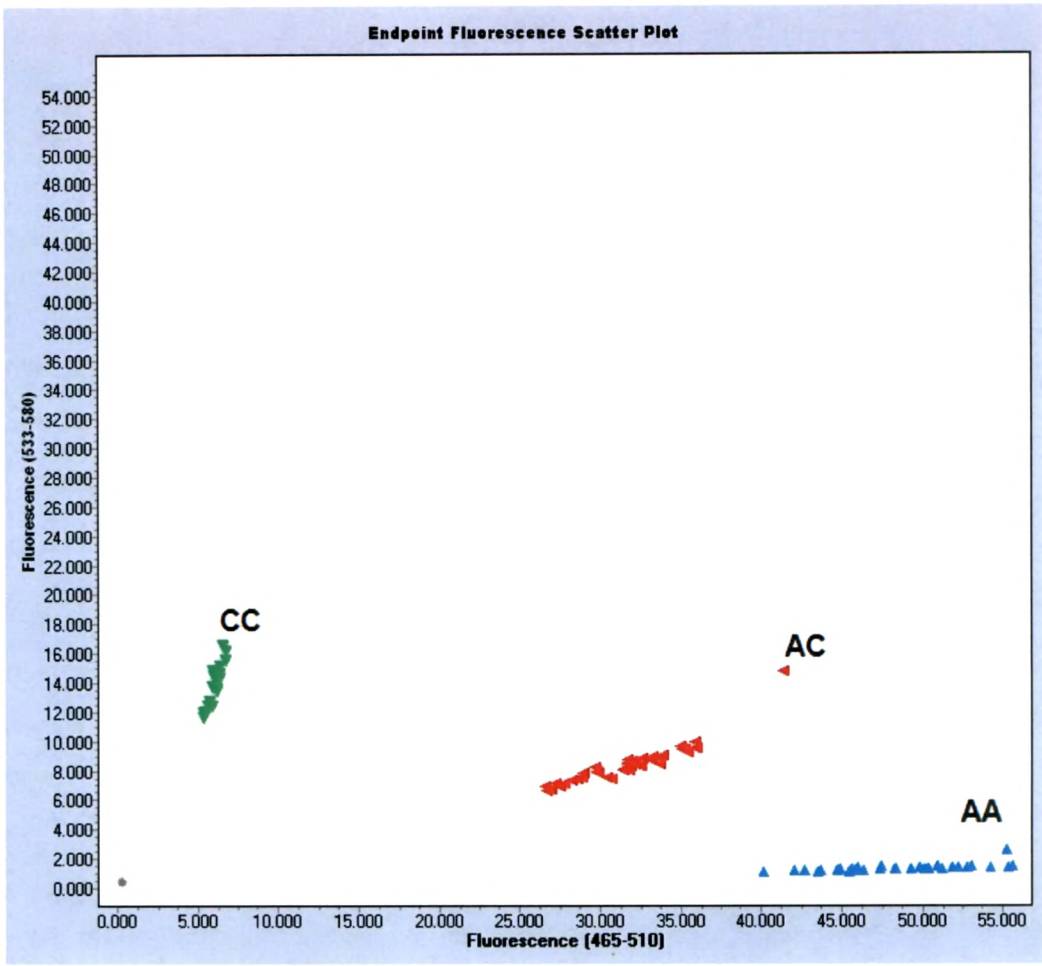


Figure 2. TaqMan end point fluoroscence analysis for *TNFB* A/C; (Thr26Asn) using dual color hydrolysis probes (FAM and VIC) by LightCycler® 480Real-Time PCR protocol. The three genotypes identified as: AA, AC and CC, based on fluorescence with Channel 465-510 (FAM for 'A' allele) and Channel 536-580 (VIC for 'C' allele). A no-template control (NTC) was used with each SNP genotyping assay (shown as grey spot).

7.3.3 Effect of *TNFB* +252G/A genotypes on age of onset of vitiligo and its progression:

When age of onset of the disease was correlated with the *TNFB* +252G/A genotypes, patients with susceptible GG genotypes showed early onset of the disease as compared to AA and GA genotypes (p=0.0001 and p=0.03 respectively) (Figure 2A). Moreover, patients with genotype GA showed early onset of the disease as compared to AA genotypes (p=0.009) (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<0.0001) (Figure 2B).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the +252G allele occurred prevalently in the group of patients with active vitiligo compared to the control group (30.00% versus 21.00%, p<0.0001) (Table 6). However, there was no statistically significant difference in the distribution of the +252G allele between patients with stable vitiligo and control group (20.00% versus 21.00%, p=0.8002) (Table 3). Interestingly, the +252G allele was more prevalent in patients with active vitiligo as compared to stable vitiligo (30.00% versus 20.00%, p=0.002) suggesting the important role of +252G allele in progression of the disease (Table 6).

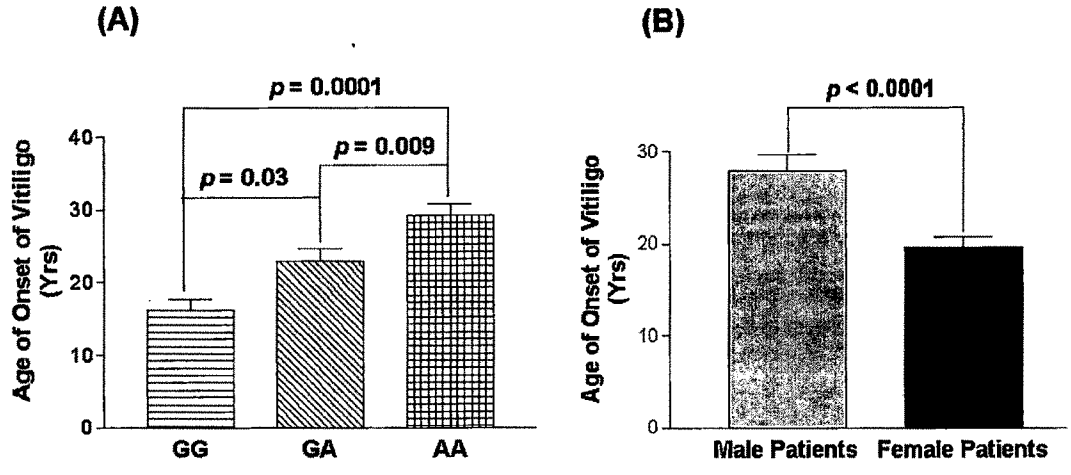


Figure 3. Age of onset of the disease in vitiligo patients:

(A) Comparison of age of onset of the disease (Years) with respect to *TNFB* +252G/A in 524 vitiligo patients. Vitiligo patients with +252GG genotype showed early age of onset of disease as compared to AA (Mean age of onset ± SEM: 16.15 ± 1.504 vs 29.23 ± 1.497; p=0.0001) and GA genotypes (Mean age of onset ± SEM: 16.15 ± 1.504 vs 22.83 ± 1.798; p=0.027). Patients with +252GA genotype showed early age of onset of disease as compared to AA genotype (Mean age of onset ± SEM: 22.83 ± 1.798 vs 29.23 ± 1.497; p=0.009).

(B) Comparison of age of onset of the disease (Years) with respect to gender differences in 224 male patients and 300 female patients with vitiligo. Female patients showed an early onset of disease as compared to male patients (Mean age of onset ± SEM: 19.63 ± 1.077 vs 27.94 ± 1.785; p<0.0001).

Table 6. Association studies for *TNFB* gene +252G/A and exon 3 A/C 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 = 393)	(n = 131)	(n = 592)			1.691[a]
	GG	40 (0.10)	6 (0.05)	29 (0.05)	0.012[a]	0.163	(1.204-
rs909253	GA	152 (0.39)	40 (0.30)	188(0.32)	< 0.0001[b]	(AV)	2.376)
(+252G/A)	AA	201 (0.51)	85 (0.65)	375(0.63)	0.945[c]		
	Allele					0.645	1.597[b]
	G	232 (0.30)	52 (0.20)	246(0.21)	0.002[a]	(SV)	(1.297-
	A	554 (0.70)	210(0.80)	938(0.79)	<0.0001[b]		1.966)
					0.8002[c]	0.389	
						(C)	0.9442[c]
							(0.6757-
							1.319)
	Genotype	(n = 393)	(n = 131)	(n = 592)			1.691[a]
	AA	40 (0.10)	6 (0.05)	29 (0.05)	0.012[a]	0.163	(1.204-
	AC	152 (0.39)	40 (0.30)	188(0.32)	< 0.0001[b]	(AV)	2.376)
rs1041981	CC	201 (0.51)	85 (0.65)	375(0.63)	0.945[c]		
(Exon 3	Allele					0.645	1.597[b]
A/C;	A	232 (0.30)	52 (0.20)	246(0.21)	0.002[a]	(SV)	(1.297-
Thr26Asn)	C	554 (0.70)	210(0.80)	938(0.79)	<0.0001[b]		1.966)
					0.8002[c]	0.389	
						(C)	0.9442[c]
							(0.6757-
							1.319)

'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.

7.3.4 Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the two polymorphisms (+252G/A and exon 3 A/C) investigated in the *TNFB* gene were in strong LD association (G/A: A/C; D' =1.00, r^2=1.00). 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 =5.24e-033) (Table 7). However, the susceptible GA haplotype was more frequently observed in vitiligo patients and increased the risk of vitiligo by 1.6-fold [*p* = 1.03e-005; odds ratio (OR): 1.635; 95% confidence interval (CI): (1.519~1.778)] (Table 7).

Table 7. Distribution of haplotype frequencies for *TNFB* gene, +252 T/C and Exon 3 C/A polymorphisms among generalized vitiligo patients and controls.

Haplotype (+252 G/A and Exon 3 A/C)	Generalized Vitiligo Patients (Freq. %) (n=426)	Controls (Freq. %) (n=592)	*p* for Association	$p_{(global)}$	Odds ratio (95% CI)
AC	274.30(0.69)	340.25(0.77)	2.13e-005	5.24e-033	0.647 [0.527~0.795]
GA	177.61(0.31)	108.62(0.23)	1.03e-005		1.635 [1.519~1.778]

CI represents Confidence Interval,

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

7.3.5 Relative gene expression of *TNFB* in patients with vitiligo and controls:

Comparison of the findings showed significant increase in expression of *TNFB* transcripts in 166 vitiligo patients compared to 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.0005) (Figure 3A). Moreover, generalized vitiligo patients showed significant higher expression of *TNFB* as compared to localized vitiligo patients (p=0.018) (Figure 3A).

The 2-ΔΔCp analysis showed approximately 0.492 fold change in the expression of *TNFB* in patients as compared to controls (Figure 3B).

7.3.6 Correlation of *TNFB* transcripts with the *TNFB* +252G/A genotypes:

Further, the expression levels of *TNFB* were analyzed with respect to +252G/A genotypes (Figure 3C). Interestingly, *TNFB* expression was significantly increased in patients with susceptible GG genotypes as compared to controls (p=0.015). Also, patients with genotypes GA showed increased *TNFB* transcripts as compared to controls (p=0.039); however, no significant difference was observed in *TNFB* expression in patients as compared to controls with AA genotypes (p=0.168) (Figure 3C).

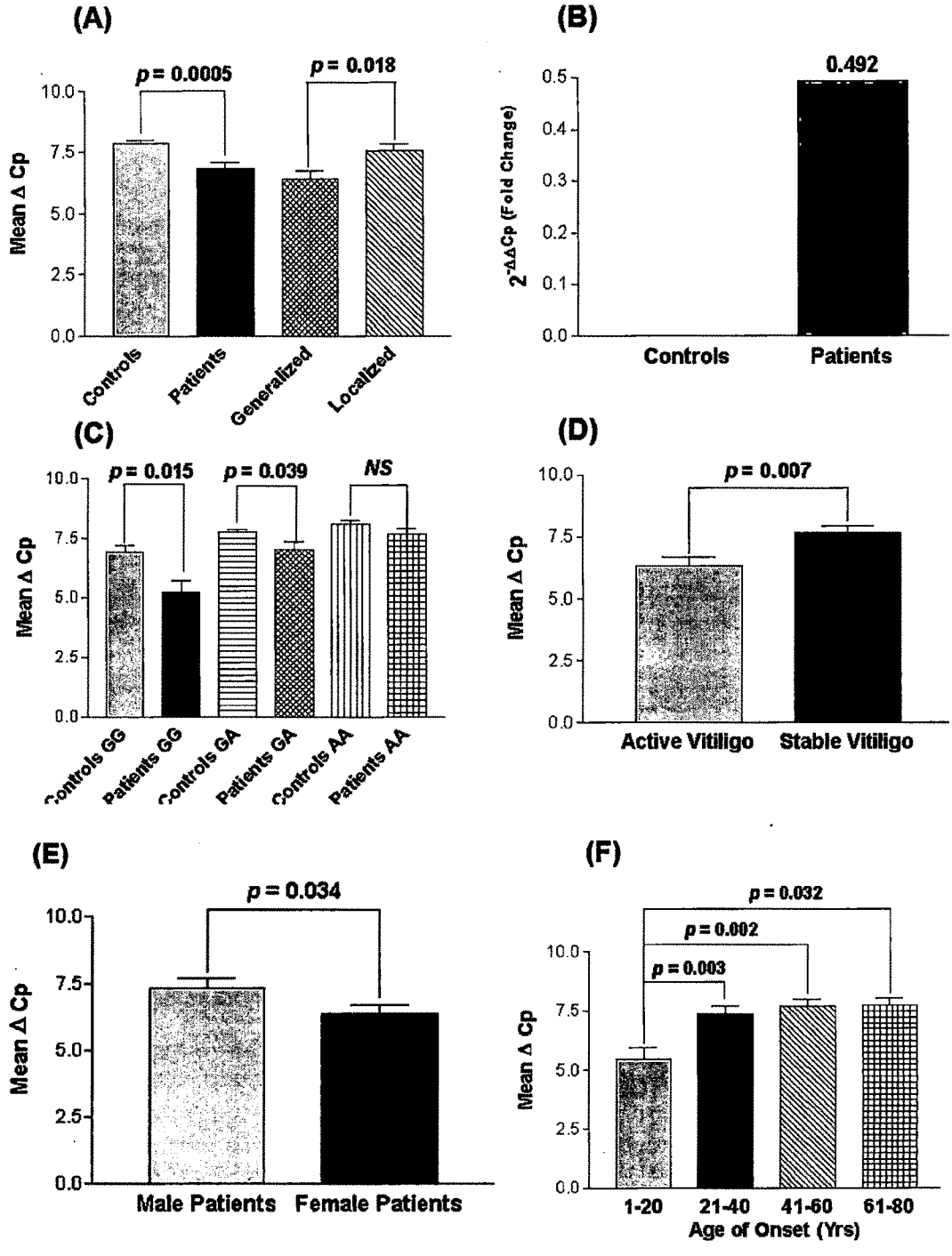
7.3.7 Correlation of *TNFB* transcripts with the *TNFB* A/C genotypes:

Further, the expression levels of *TNFB* were also analyzed with respect to **A/C** genotypes (Figure 3G). Interestingly, *TNFB* expression was significantly increased in patients with susceptible AA genotypes as compared to controls (p=0.015). Also, patients with genotypes AC showed increased *TNFB* transcripts as compared to controls (p=0.039); however, no significant difference was observed in *TNFB* expression in patients as compared to controls with CC genotypes (p=0.168) (Figure 3G).

7.3.8 Effect of *TNFB* expression on disease progression:

In addition, we also checked the effect of *TNFB* expression on progression of the disease i.e. active and stable cases of vitiligo (Figure 3D). Interestingly, active vitiligo patients showed significant increase in expression of *TNFB* transcripts as compared to the patients with stable vitiligo (p=0.007) suggesting the involvement of *TNFB* 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 *TNFB* expression as compared to male patients (p=0.034) (Figure 3E).

When *TNFB* expression was monitored in different patient groups of age of onset, patients with the age group 1-20 yrs showed significant increase in expression of *TNFB* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.003, p=0.002 and p=0.032 respectively) suggesting the importance of *TNFB* in early onset of the disease (Figure 3F).



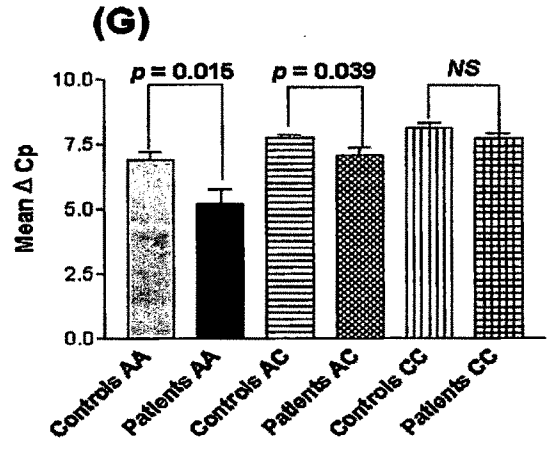


Figure 4. Relative gene expression of *TNFB* in controls and vitiligo patients:

(A) Expression of *TNFB* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significant increase in mRNA levels of *TNFB* as compared to controls (Mean ΔCp ± SEM: 6.830 ± 0.2435 vs 7.853 ± 0.1244; p=0.0005). Generalized vitiligo patients showed significant increase in mRNA levels of *TNFB* as compared to localized patients (Mean ΔCp ± SEM: 6.372 ± 0.3498 vs 7.549 ± 0.2516; p=0.018).

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

(C) Expression of *TNFB* transcripts with respect to *TNFB* +252G/A in 166 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significant increase in mRNA levels of *TNFB* with GG (Mean ΔCp ± SEM: 5.217 ± 0.5087 vs 6.918 ± 0.2808; p=0.015) and GA (Mean ΔCp ± SEM: 7.028 ± 0.3376 vs 7.761 ± 0.0952; p=0.039) genotypes as compared to controls. There was no significant difference in the expression of *TNFB* with AA genotypes (Mean ΔCp ± SEM: 7.678 ± 0.2379 vs 8.102 ± 0.1747; p=0.168) as compared to controls. [*NS* = non-significant]

(D) Expression of *TNFB* 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 significant increase in mRNA levels of *TNFB* as compared to stable vitiligo patients (Mean ΔCp ± SEM: 6.314 ± 0.338 vs 7.676 ± 0.2629; p=0.007).

(E) Expression of *TNFB* 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 significant increase in mRNA levels of *TNFB* as compared to male vitiligo patients (Mean ΔCp ± SEM: 6.343 ± 0.3494 vs 7.374 ± 0.3165; p=0.034).

(F) Expression of *TNFB* 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 significant increase in expression of *TNFB* mRNA as compared to the age groups 21-40 yrs (Mean ΔCp ± SEM: 5.482 ± 0.4821 vs 7.364 ± 0.3224; p=0.003), 41-60 (Mean ΔCp ± SEM: 5.482 ± 0.4821 vs 7.689 ± 0.3293; p=0.002) and 61-80 yrs (Mean ΔCp ± SEM: 5.482 ± 0.4821 vs 7.743 ± 0.2944; p=0.032).

(G) Expression of *TNFB* transcripts with respect to *TNFB* A/C in 166 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significant increase in mRNA levels of *TNFB* with AA (Mean ΔCp ± SEM: 5.217 ± 0.5087 vs 6.918 ± 0.2808; p=0.015) and AC (Mean ΔCp ± SEM: 7.028 ± 0.3376 vs 7.761 ± 0.0952; p=0.039) genotypes as compared to controls. There was no significant difference in the expression of *TNFB* with CC genotypes (Mean ΔCp ± SEM: 7.678 ± 0.2379 vs 8.102 ± 0.1747; p=0.168) as compared to controls. [*NS* = non-significant]

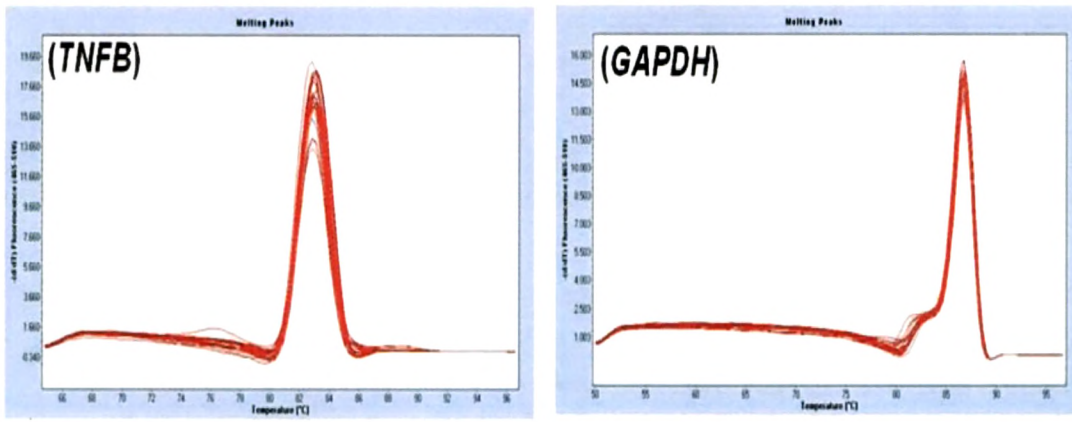


Figure 5. Melt curve analysis of *TNFB* and *GAPDH* showing specific amplification.

7.4 DISCUSSION

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. TNF plays a central role in the so-called ''cytokine storm'' characteristic of several autoimmune diseases. Since many aspects of the cellular and humoral immune responses are under genetic control and account for individual differences in immune response patterns, there is increasing interest in genetic polymorphisms that affect inflammatory cytokines, which might explain the well-known diversity among clinical findings and outcomes in critically ill patients affected by the same disease.

TNFA and *TNFB* polymorphisms are reported to be associated with the inflammatory and immunomodulatory responses and are involved in the modulation of gene expression, and thus affect the precipitation and progression of the diseases (Kaluza *et al.*, 2000; Wilson *et al.*, 1997; Gonza′ lez *et al.*, 2003; Messer *et al.*, 1991; Ozaki *et al.*, 2002).

The polymorphism (A→G) of the *TNFB* gene is present in intron 1 at position +252 (Messer *et al.*, 1991). Although the *TNFB* +252 G/A polymorphic site is located in an intron, it is linked to an amino acid substitution at position 26 of the TNFβ protein, the substitution being conserved as asparagine in the *TNFB* +252G and as threonine in the *TNFB* +252A allele (Messer *et al.*, 1991). The *TNFB* +252G allele has been shown to be associated with increased expression and the *TNFB* +252A allele with decreased production of the TNFβ protein (Messer *et al.*, 1991).

The *TNFA* -308A and the *TNFB* +252G alleles are constituents of one of the extended ancestral haplotypes (AH8.1) located in the chromosomal region 6p21.3-21.1 (MHC). This haplotype is known to be associated with serious disorders of the immune system (Dawkins *et al.*, 1983; Candore *et al.*, 2002; Candore *et al.*, 2003). The *TNFB* +252G allele was also associated with several autoimmune diseases such as type 1 diabetes mellitus (Dawkins *et al.*, 1983; Aly *et al.*, 2006) and systemic lupus erythematosus (Smerdel-Ramoya *et al.*, 2005; McHugh *et al.*, 2006). The present study for the first time reports significant association of *TNFB*+252G/A polymorphism with vitiligo susceptibility. In particular, we found that +252G allele was prevalent in generalized

vitiligo patients as compared to localized vitiligo patients and healthy controls. We also found that +252G allele was prevalent in active cases of vitiligo as compared to patients with stable vitiligo and controls suggesting the involvement of the allele in progression of the disease. Moreover, +252G allele was significantly associated with female patients as compared to male patients suggestive of the fact that females are more prone to autoimmune diseases. The age of onset analysis further suggested that patients with GG genotype had an early onset of the disease as compared to patients with GA and AA genotypes indicating the important role of +252G allele in disease susceptibility.

The whole *TNFB* gene is in strong linkage disequilibrium, therefore the +252G allele naturally coexists with the 804A allele of exon 3 A/C polymorphism (Thr26Asn) (Clarke *et al.*, 2006). Ozaki *et al.*, investigated the functionality of the +252G/A and 804A/C SNPs in the *TNFB* gene (Ozaki *et al.*, 2002). The 804A/C polymorphism causes an amino-acid change from threonine (T) to asparagine (N) at codon 26. They found that the variant protein 26N is associated with a two fold increase in the induction of cell adhesion molecules in vascular smooth muscle cells (Ozaki *et al.*, 2002). We found that *TNFB* exon 3 A/C polymorphism (Thr26Asn) was significantly associated with vitiligo susceptibility in Gujarat population. In particular, allele 'A' was prevalent in generalized vitiligo patients as compared to localized vitiligo patients and healthy controls. We also found that 'A' allele was prevalent in active cases of vitiligo as compared to patients with stable vitiligo and controls suggesting the involvement of the 'A' allele in progression of the disease. Moreover, the 'A' allele was significantly associated with female patients as compared to male patients suggestive of the fact that females are more prone to autoimmune diseases. The age of onset analysis further suggested that patients with AA genotype had an early onset of the disease as compared to patients with AC and CC genotypes indicating the important role of 'A' allele in disease susceptibility.

It has been reported that cytokines such as IFNγ, TNFα and TNFβ can initiate apoptosis and thus lead to melanocyte death in the context of autoimmunity (Huang *et al.*, 2002). TNFα/β also has the capacity to inhibit melanogenesis through an inhibitory effect on tyrosinase and tyrosinase related protein (Martinez-Esparza *et al.*, 1998). In addition, IFNγ and TNFα/β 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 resulting in destruction of melanocytes in vitiligo (Al Badri, 1993; Morelli and Norris, 1993).

Our study also confirmed that the *TNFB* mRNA levels have increased significantly in vitiligo patients as compared to controls. Our recent study also suggested an increase in *ICAM1* mRNA levels in vitiligo patients compared to controls. Furthermore, in patients with early age of onset had increased *ICAM1* expression as compared with patients with late onset of disease. These results are in concordance with the previous reports (Reimann *et al.*, 2012; Ahn *et al.*, 1994; al Badri *et al.*, 1993; Yagi *et al.*, 1997). Therefore, ICAM1 is probably an important link between cytokines and T cells involved in vitiligo pathogenesis.

The *TNFB* +252 G/A and Thr26Asn A/C SNPs gene could affect susceptibility/ resistance to vitiligo through its influence on the production of TNFα and TNFβ. As *TNFA* and *TNFB* genes are tandemly arranged within the HLA complex, and it has been shown that the *TNFB* gene polymorphisms influence the level of production of the TNFα protein. Studies indicate that variation at both *TNFA* -308 G/A and *TNFB* +252 G/A can affect TNFα production levels (Abraham and Kroeger, 1999; Ozaki *et al.*, 2002).

Pociot *et al.*, (1993) showed that secretion of TNFα was shown to be the highest in *TNFB* +252GG homozygotes, the lowest in the *TNFB* +252AA homozygotes, and intermediate in the *TNFB* +252GA heterozygous individuals. *In vitro*, direct analysis of skin T cells from margins of vitiliginous skin showed that polarized type-1 T cells ($CD4^+$ and particularly $CD8^+$), which predominantly secrete IFNγ 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 (Grimes *et al.*, 2004; Moretti *et al.*, 2002), IL6 (Moretti *et al.*, 2002), IFNγ (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. Since elevated levels of TNFα have been associated with vitiligo, this could explain the association of *TNFB*

+252G allele with susceptibility to vitiligo in the present study. Homozygosity for the *TNFB* +252A allele will be protective because of its association with lower TNFα/β levels. Previously, Hasegawa *et al.*, also suggested the correlation of *TNFB* +252GA polymorphism with elevated levels of TNFα in pulmonary fibrosis in scleroderma (Hasegawa *et al.*, 1997). *TNFB* +252GG homozygosity has also been shown to be associated with susceptibility to systemic lupus erythematosus in some populations from Germany, Korea, and China (Bettinotti *et al.*, 1993; Kim *et al.*, 1996; Lee *et al.*, 1996; Zhang *et al.*, 1997).

The variant of *TNFB* gene present in exon 3 leads to the substitution Asp to Thr at the amino acid position 26 (Kobayashi *et al.*, 1986) and correlated with an altered level of TNFβ production (Messer *et al.*, 1991). The exon 3 Asp to Thr is in strong linkage disequilibrium with +252G/A polymorphism of intron 1 and combination of these allelic forms may lead to the different levels of TNF production in response to various physiological and pathological stimuli (Steinman, 1995) and in turn might result in predisposition to the development of vitiligo and involved in the different clinical aspects of the disease. Evidence implicating the polymorphism in the *TNFB* gene (T26N) with increased susceptibility to T2DM has emerged from Japanese and Danish studies (Yamada *et al.*, 2004; Hamid *et al.*, 2005). In the present study we also found that +252G/A and exon 3 A/C SNPs were in strong LD association. The susceptible GA haplotype was more frequently observed in vitiligo patients as compared to controls (31% vs 23%) and increased the risk of vitiligo by 1.6-fold.

There are no reports available for gene expression analysis of *TNFB* in vitiligo patients hence the present study also focused on the *TNFB* expression in vitiligo patients. Our results clearly suggest the important role of *TNFB* in pathogenesis of vitiligo. Vitiligo patients showed significant increase in *TNFB* transcripts levels as compared to controls suggesting that melanocyte death in patients could be triggered due to the increased *TNFB* levels. For the first time we report that generalized vitiligo has significantly higher *TNFB* transcript 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 *TNFB* transcript levels as compared to the patients with stable vitiligo which signifies the role of *TNFB* in disease progression. Our results also suggest that there are

significantly higher transcript levels of *TNFB* 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; Panchanathan and Choubey, 2012; Afshan *et al.*, 2012). Furthermore, the genotype-phenotype analysis for +252G/A polymorphism indicated that patients with GG and GA genotypes had higher expression of *TNFB* transcripts suggesting the crucial role of +252G allele in pathogenesis of vitiligo. Also, the genotype-phenotype analysis of *TNFB* exon 3 A/C showed that patients harboring AA and AC genotypes had higher expression of *TNFB* suggesting that allele 'A' plays an important role in increased expression of *TNFB*.

In addition, such gene polymorphisms might provide useful biomarkers for the screening of patients at risk, as well in the identification of those most likely to benefit from specific therapeutic choices (Marshall and Reinhart, 2009).

In conclusion, our findings suggest that the increased *TNFB* levels in vitiligo patients could result, at least in part, from variations at the genetic level which in turn leads to increased *ICAM1*. For the first time, we show that the +252G/A polymorphism of the *TNFB* gene influences the *TNFB* expression levels in vitiligo. The study also emphasizes the influence of *TNFB* on the disease progression, onset of the disease and gender biasness for developing vitiligo. More detailed studies regarding the role of *TNFB* in precipitation of vitiligo and the development of effective anti-TNF agents may prove to be useful as preventive/ameliorative therapies.

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