CHAPTER IV

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

4.1 INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

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

4.2 MATERIALS AND METHODS

4.2.1 Study Subjects:

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

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

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

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

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

4.2.2 Blood collection and DNA extraction:

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

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

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

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

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

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

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

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

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

4.2.5.1 RNA extraction and cDNA synthesis:

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

4.2.5.2 Real-time PCR:

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

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

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

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

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

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

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

4.2.7 Statistical analysis:

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

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

4.3 RESULTS

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

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

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

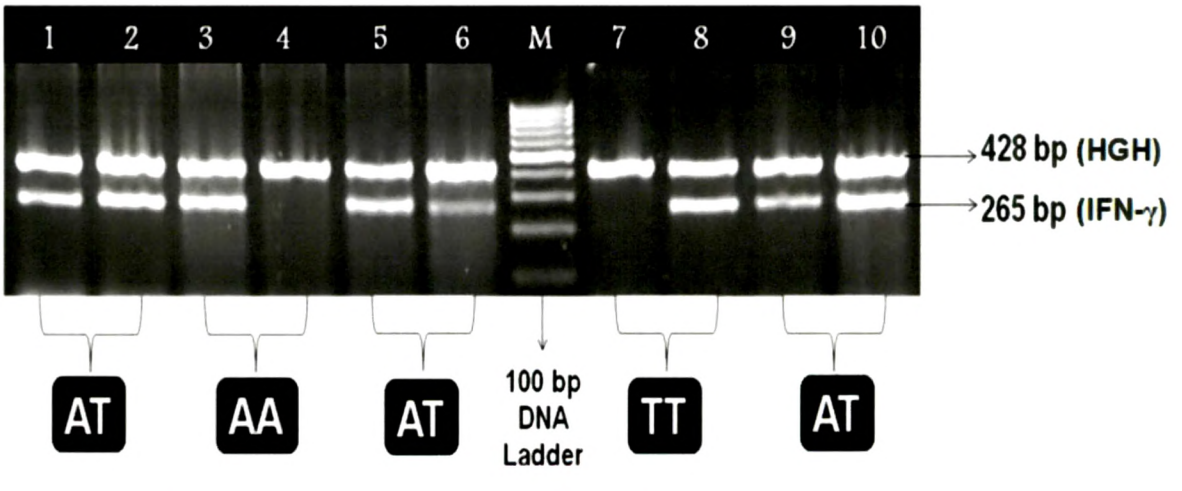


Figure 1. Representative gel picture for genotyping of *IFNG* +874A/T polymorphism by ARMS-PCR on 2.0% agarose gel electrophoresis: lane M: 100 bp DNA Ladder; lanes 3 and 4: AA (homozygous-low producer) genotype; lanes 1, 2, 5, 6, 9 and 10: AT (heterozygous-medium producers) genotypes; lanes 7 and 8: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

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

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

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

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

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

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

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

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

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

'n' represents number of Patients,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

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

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

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

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

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

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

'n' represents number of Patients,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

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

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

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

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

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

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

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

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

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

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

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

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

NS= not significant.

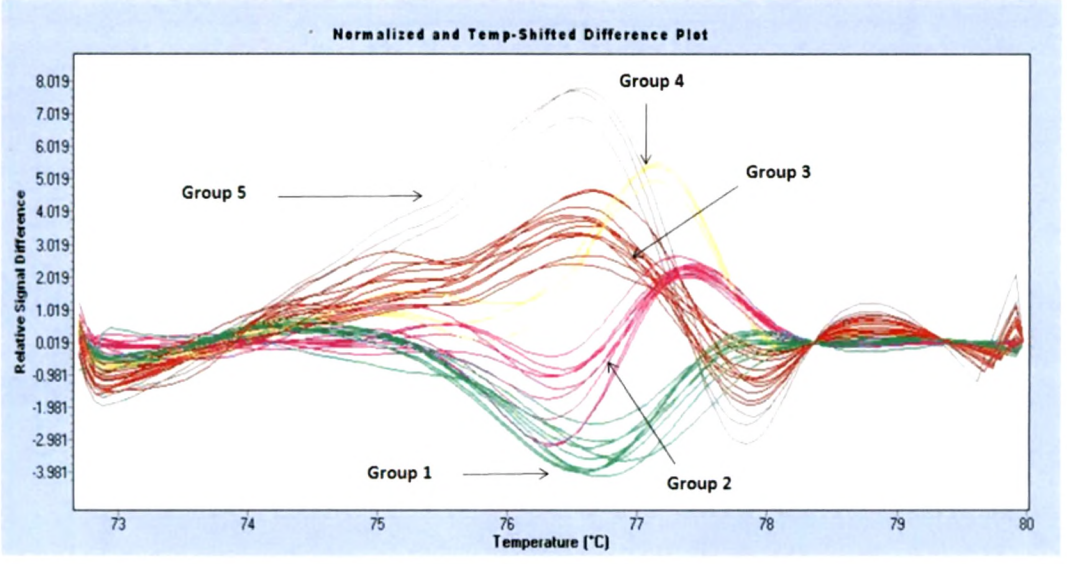
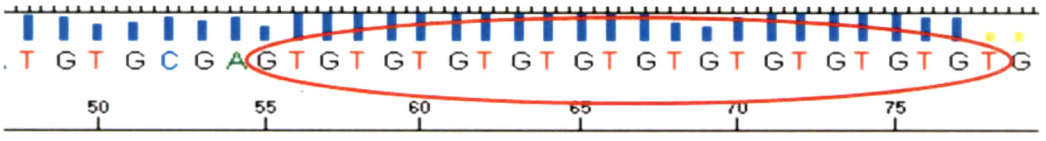
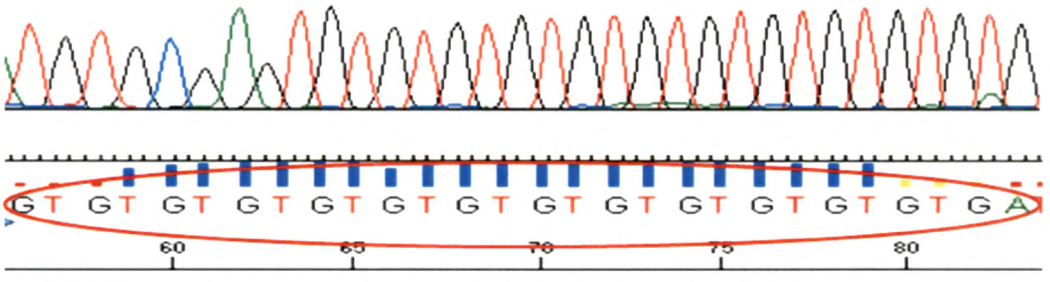


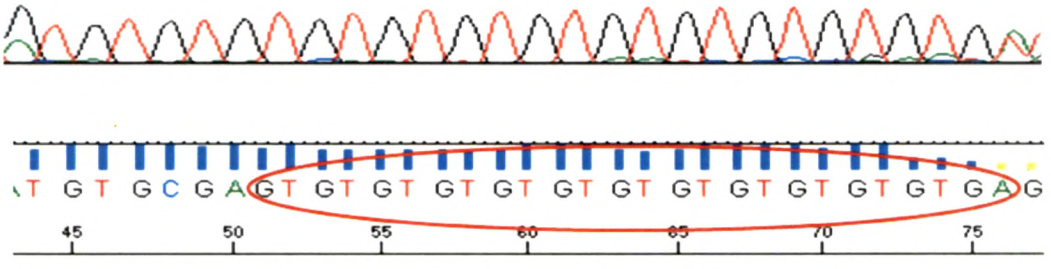
Figure 2. (A) High Resolution Melt (HRM) curve analysis for *IFNG* CA microsatellite (rs3138557) polymorphism using LightCycler®480 Real-Time PCR protocol. The five genotypes identified as: 12-12 CA, 13-13 CA, 12-13 CA, 13-14 CA and 12-14 CA, based on normalized and temperature shifted difference plots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



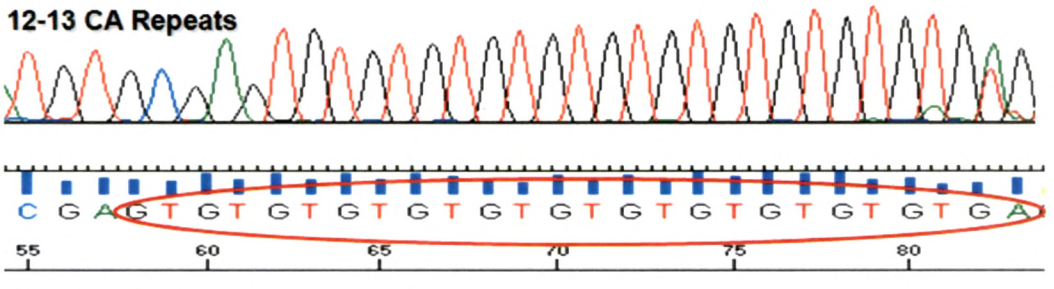
12 CA Repeats



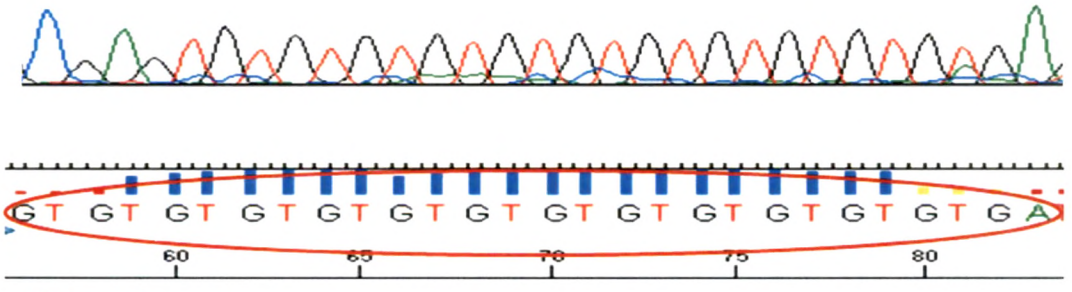
13-14 CA Repeats



12-13 CA Repeats



13 CA Repeats



12-14 CA Repeats

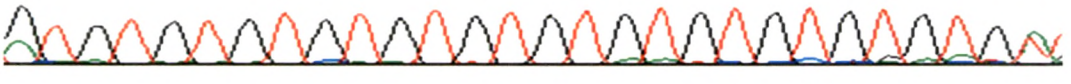


Figure 2. **(B)** DNA sequencing of samples from each representative groups obtained by HRM analysis. Different *IFNG* CA repeats obtained are shown in electrophoretograms as: 12-12 CA, 13-14 CA, 12-13 CA, 13-13 CA and 12-14 CA repeats.

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

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

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

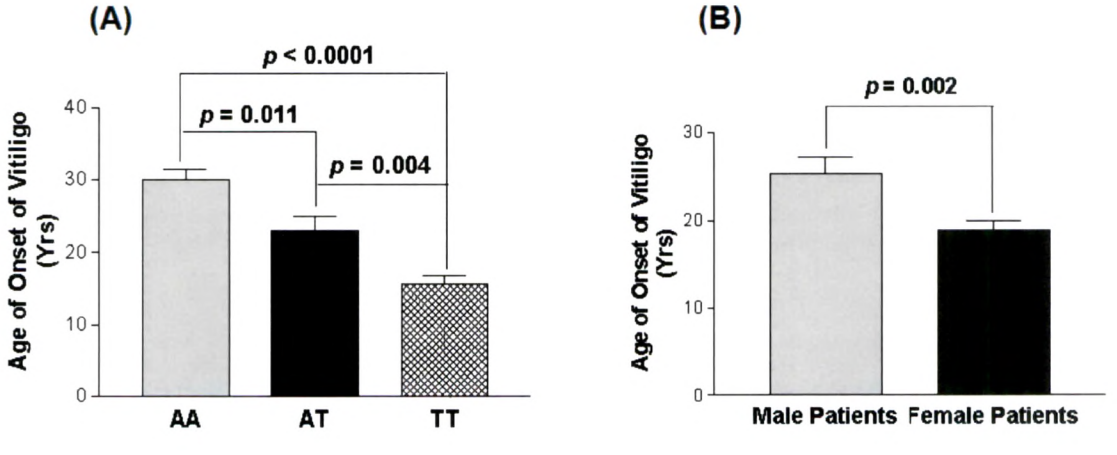


Figure 3. **Age of onset of the disease in vitiligo patients:**

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

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

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

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

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

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

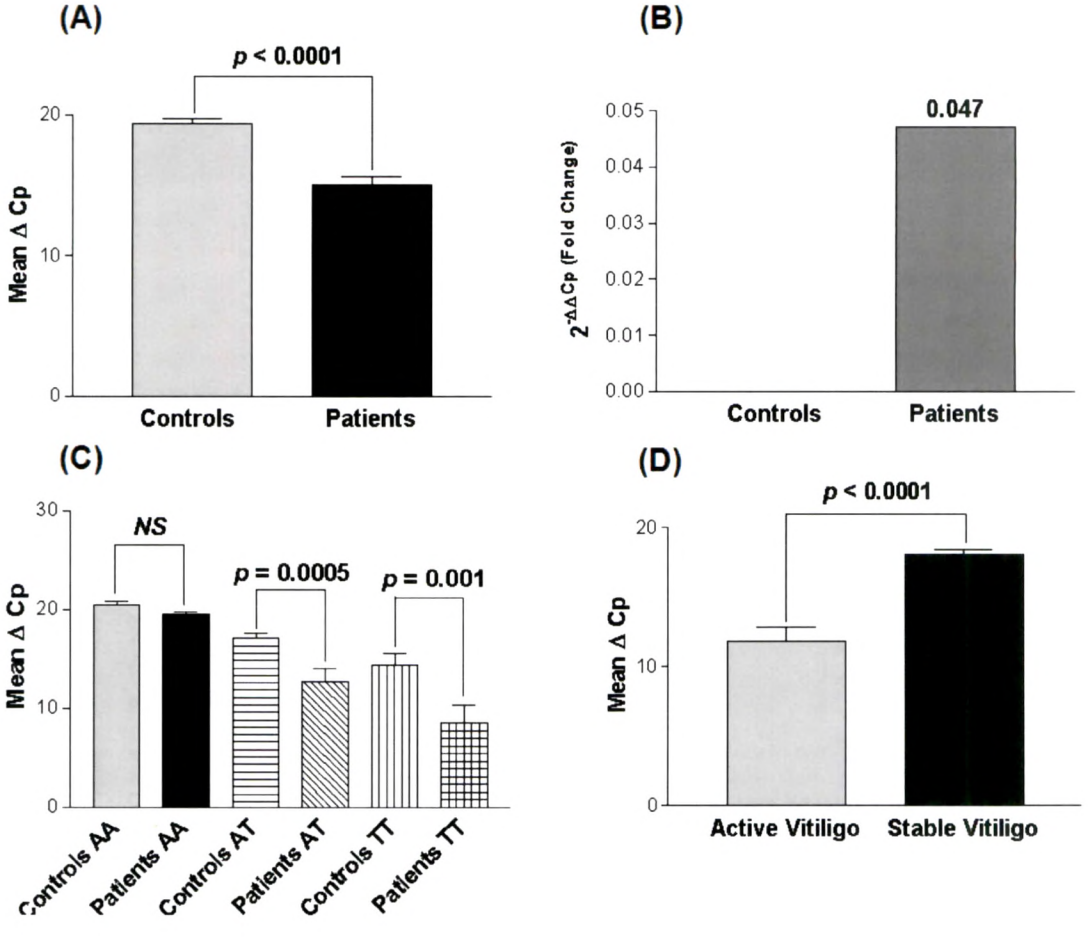
Further, the expression levels of *IFNG* were analyzed with respect to +874 A/T (rs2430561) genotypes (Figure 4C). Interestingly, *IFNG* expression was significantly increased in patients with susceptible TT genotypes as compared to controls ($p=0.001$). Also, patients with genotypes AT showed increased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

5.3.6 Effect of *IFNG* expression on disease progression:

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

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

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



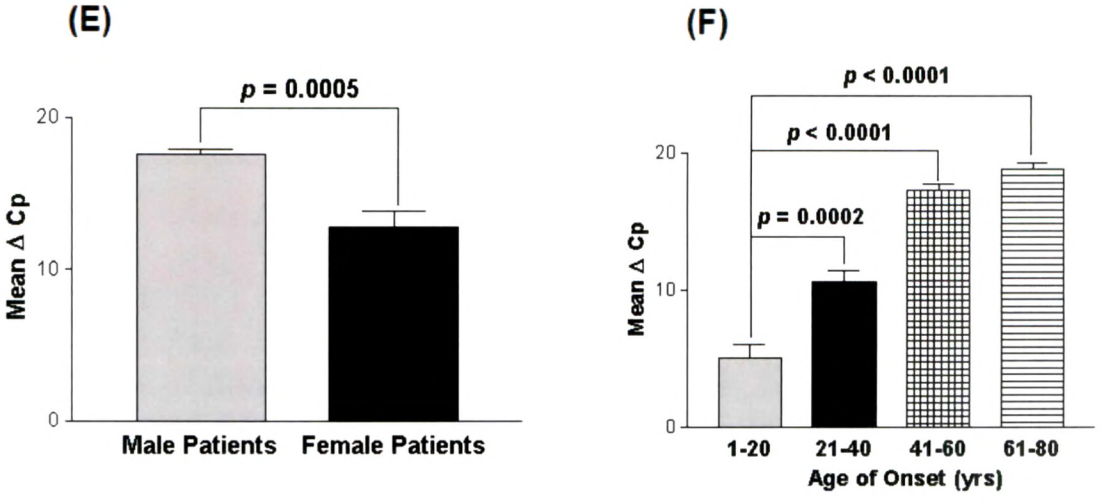


Figure 4. Relative gene expression of *IFNG* in controls and vitiligo patients:

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

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

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

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

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

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

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

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

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

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

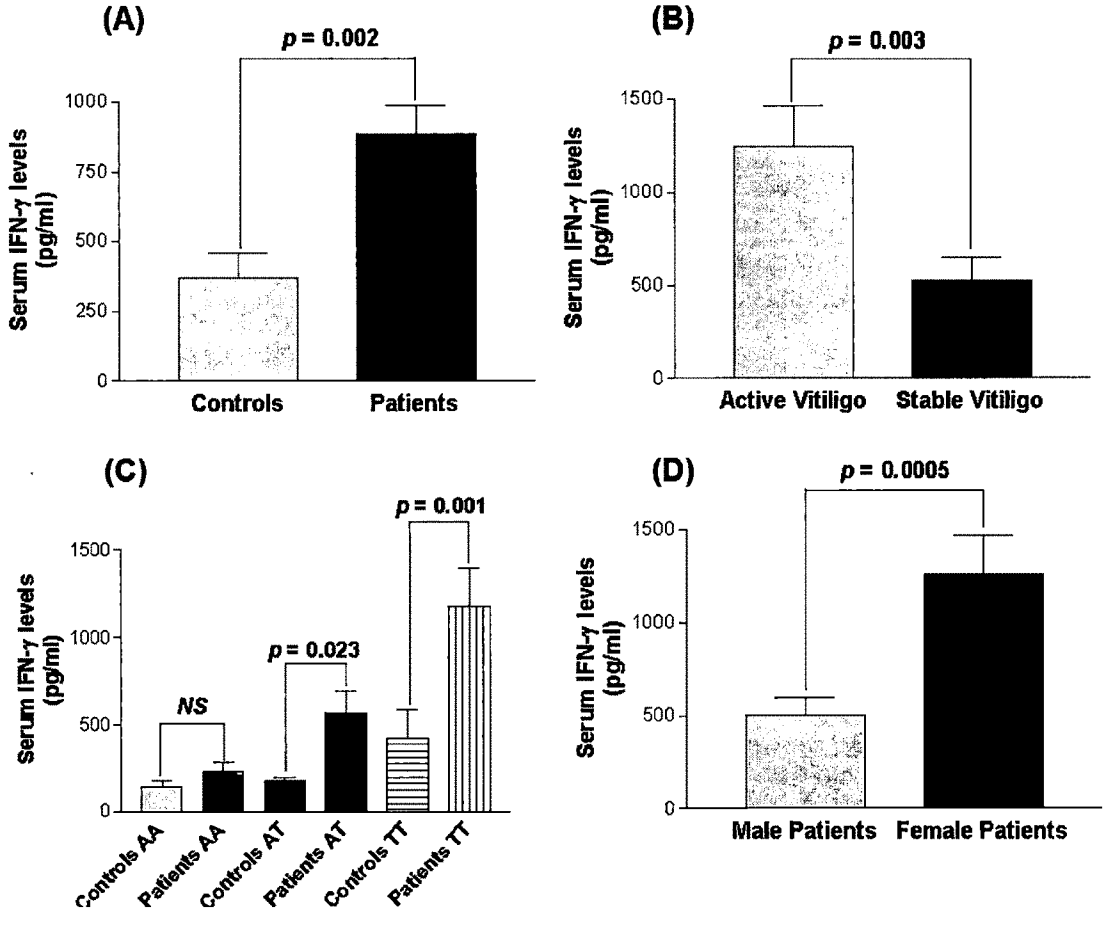


Figure 5. Serum IFNγ levels in controls and vitiligo patients:

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

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

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

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

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

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

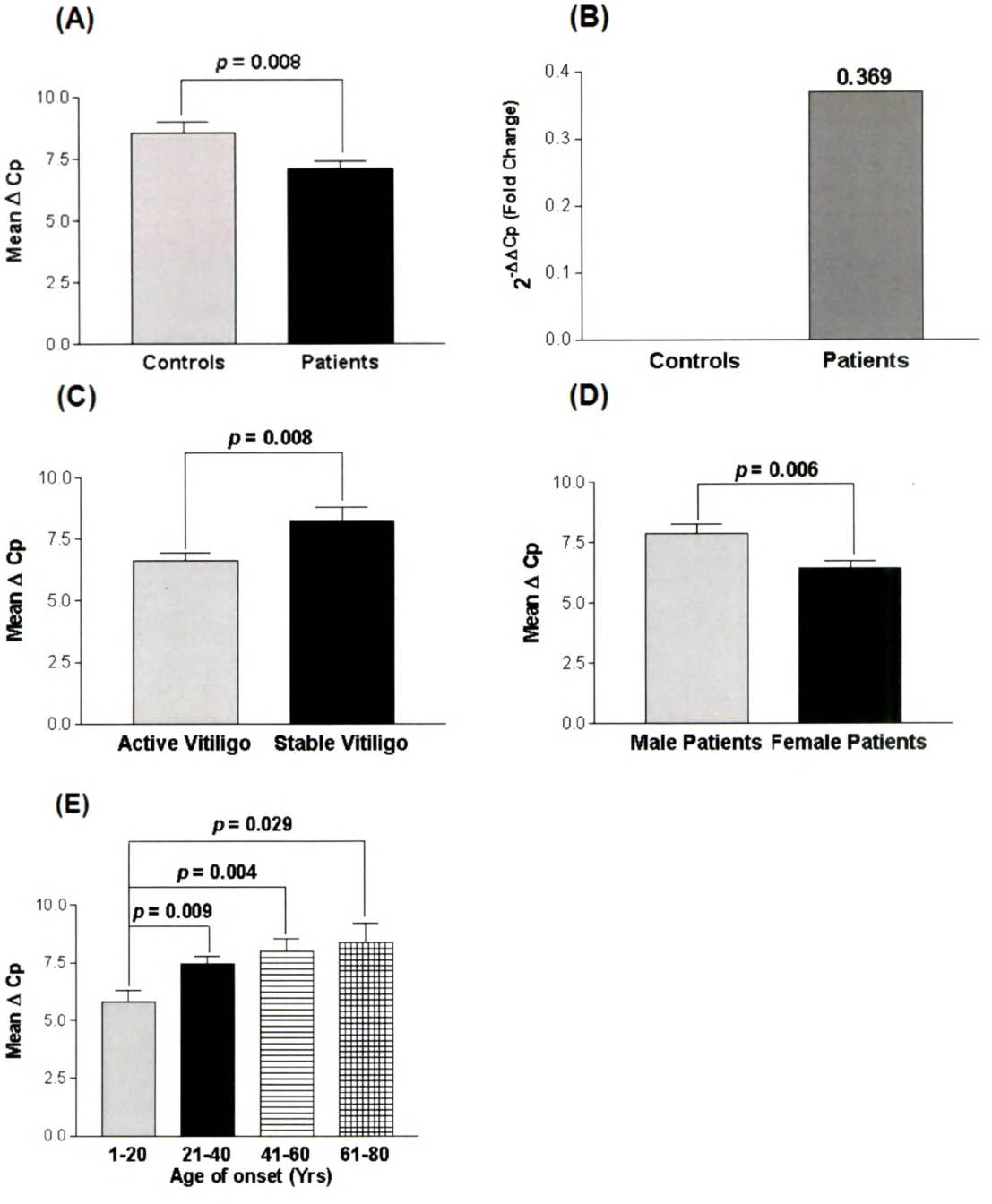


Figure 7. Relative gene expression of *ICAM1* in controls and vitiligo patients:

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

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

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

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

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

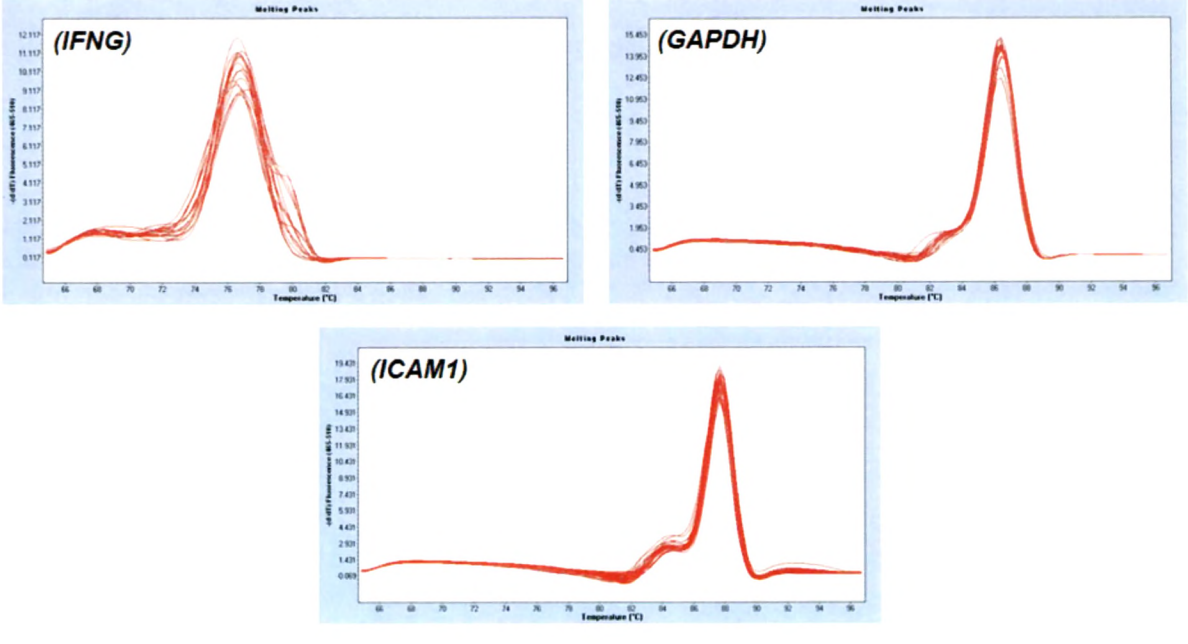


Figure 8. Melt curve analysis of *IFNG*, ICAM1 and *GAPDH* showing specific amplification.

4.4 DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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