Involvement of Interferon-Gamma Genetic Variants and Intercellular Adhesion Molecule-1 in Onset and Progression of Generalized Vitiligo

Mitesh Dwivedi,[1] Naresh C. Laddha,[1] Kriti Shah,[1] Bela J. Shah,[2] and Rasheedunnisa Begum[1]

Interferon-gamma (IFN-γ) is a paracrine inhibitor of melanocytes and genetic variability due to intron 1 polymorphisms in *IFNG* has been reported to be associated with increased risk for several autoimmune diseases. The aim of present study was to determine whether intron 1 +874A/T (rs2430561) and CA microsatellite (rs3138557) polymorphisms in *IFNG* are associated with generalized vitiligo (GV) susceptibility and expression of *IFNG* and intercellular adhesion molecule-1 (*ICAM1*) affects the disease onset and progression. Here we report that *IFNG* CA microsatellite but not +874A/T may be a genetic risk factor for GV; however, +874T allele plays a crucial role in increased expression of *IFNG* mRNA and protein levels which could affect the onset and progression of the disease. Active GV patients showed increased IFNG levels compared to stable GV patients. The genotype-phenotype analysis revealed that *IFNG* expression levels were higher in patients with +874 TT genotypes and 12 CA repeats. Patients with the early age of onset showed higher *IFNG* expression and female GV patients showed higher *IFNG* and *ICAM1* expression implicating gender biasness and involvement of IFN-γ in early onset of the disease. Moreover, the increased IFN-γ levels in patients lead to increased *ICAM1* expression, which could be a probable link between cytokines and T-cell involvement in pathogenesis of GV.

Introduction

GENERALIZED VITILIGO (GV) is an acquired, noncontagious disorder in which progressive loss of pigmentation from skin, overlying hair, and oral mucosa results from autoimmune loss of melanocytes (Nordlund and others 2006). It is a polygenic, multifactorial disorder involving multiple susceptibility genes and unknown environmental triggers (Majumder and others 1993; Nath and others 1994; Sun and others 2006). It affects ∼0.5%–1% of the world population (Taieb and others 2007). The exact etiology of vitiligo remains obscure, but autoimmunity has been strongly implicated in GV, because ∼30% of vitiligo patients are affected with at least one additional autoimmune disorder (Alkhateeb and others 2003; Laberge and others 2005). Epidemiological studies have shown frequent family clustering of vitiligo cases, with elevated risk of vitiligo in first-degree relatives and high concordance in monozygotic twins (Alkhateeb and others 2003; Sun and others 2006) suggesting a genetic basis for vitiligo. A number of genes that are involved in regulation of immunity, including *MHC*, *ACE*, *CAT*, *CTLA4*, *COMT*, *ESR*, *MBL2*, *PTPN22*, *HLA*, *NALP1*, *XBP1*, *FOXP1*, and *IL2RA* have been implicated in the pathogenesis of GV (Spritz 2007, 2008, 2010). However, the

PRO2268 gene has been identified as a novel susceptibility locus for vitiligo (Douroudis and others 2011), and mapped to the 12q14 chromosomal region, which harbors the genes encoding interferon-gamma (*IFNG*), interleukin (*IL*)-26 and *IL*-22, whose gene products play key roles in immune signaling.

More than 30 susceptibility, loci have been identified for GV on the basis of genetic linkage and Genome-wide association studies (GWAS) (Jin and others 2012). All but one of these genes encodes proteins involved in regulation of the immune system and/or have been genetically associated with susceptibility to other autoimmune diseases. The sole exception is TYR, encoding tyrosinase, the key enzyme of melanin biosynthesis and the principal vitiligo autoimmune antigen. These genes together account for a relatively small fraction of the genetic risk factors of GV, indicating that many additional vitiligo susceptibility genes undoubtedly remain to be discovered. Moreover, studies by Birlea and others (2013) and Singh and others (2012) in Indian population, support the association of GV with loci in the MHC class II region; however, GWAS in the EUR population vitiligo show primary association with HLA-A in the distal class I region (Jin and others 2010, 2011) in addition to the association of multiple signals in the MHC class II region.

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[1]Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, India.
[2]Department of Dermatology, STD and Leprosy, B.J. Medical College and Civil Hospital, Ahmedabad, India.

Additionally, GWAS studies in the Chinese population show principal MHC association in the class III region (Quan and others 2010) and in the proximal class I region, between HLA-B and HLA-C (Liu and others 2012). These differences and similarities in MHC genetic association in different populations may partly responsible for the differing prevalence of this autoimmune disease in different groups around the world.

Cytokines are small immune-regulatory molecules which can generate an inappropriate immune response due to their imbalance (Feldmann and others 1998). Moretti and others (2002a, 2002b) have shown cytokine imbalance in skin of vitiligo patients suggesting their role in autoimmunity. Interferon-gamma (IFN-γ, a type II interferon) is a pleiotropic cytokine and is a key regulator of immune system (Schroder and others 2004). The principal sources of IFN-γ are helper T-cells and natural killer cells, although many other cells can produce it, including keratinocytes. Apart from host defense, IFN-γ also contributes to autoimmune pathology. IFN-γ is suggested to cause induction of antimelanocyte antibodies or activation of cytotoxic T-cells (Miller and others 2002; SeÈkin and others 2004). It has been proposed that melanocyte death is mediated by apoptosis in the context of autoimmunity, and cytokines, such as IFN-γ and tumor necrosis factor (TNF)-α can initiate apoptosis (Huang and others 2002). Additionally, IFN-γ and TNF-α induce the expression of intercellular adhesion molecule-1 (ICAM1) on the cell-surface of melanocytes (Yohn and others 1990). Increased expression of ICAM1 on the melanocytes enhances T cell- melanocyte attachment in the skin and may be involved in the destruction of melanocytes in vitiligo (Al Badri 1993). Kocer and others (2009) suggested that IFN-γ therapy may cause vitiligo. These reports signify that IFN-γ may play a crucial role in vitiligo susceptibility. Therefore, it is possible that individuals who naturally produce higher levels of IFN-γ will exhibit different susceptibility, or severity towards GV.

IFNG gene is located on human chromosome 12q24 spanning ∼5.4 kb, consisting of 4 exons and 3 introns (Gray and Goeddel 1982; Naylor and others 1983). The *IFNG* coding region is invariant, with no reported polymorphisms (Hayden and others 1997). However, there are 2 well-known single nucleotide polymorphisms (SNPs) in the *IFNG* gene noncoding region (intron 1): [+874A/T polymorphism (rs2430561); CA microsatellite (rs3138557)]. Allele 2, with 12 CA repeats is associated with *in vitro* constitutive high IFN-γ production. In addition, +874A/T SNP at the 5′ end of the CA repeat region has been correlated with the presence or absence of the microsatellite allele 2 (Pravica and others 1999). Also, the presence of *IFNG* (+874A^{lo}/T^{hi}) polymorphism creates a putative nuclear factor-κB (NF-κB) binding site and shows preferential binding to the T allele and correlates with high IFN-γ producer phenotype (Pravica and others 2000).

In the present study, we have made an attempt to understand the role of IFN-γ and ICAM1 in pathogenesis of GV. Hence, the objectives of this study were, (1) to determine whether the intron 1 polymorphisms of *IFNG* [+874A/T (rs2430561) and 5′ end CA microsatellite (rs3138557)] are associated with GV susceptibility; (2) to measure and compare *IFNG* and *ICAM1* transcripts and serum IFN-γ levels in GV patients and controls; (3) to correlate *IFNG* polymorphisms/levels with onset and progression of the disease.

Materials and Methods

Study subjects

The study group included 517 GV patients (including acrofacial vitiligo and vitiligo universalis) who referred to S.S.G. Hospital, Vadodara, India and B.J. Medical College and Civil hospital, Ahmedabad, India (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/jir). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune diseases.

The patients were divided into 2 groups based on whether the existing lesions were spreading and/or new lesions had appeared within the previous 6 months: an affirmative answer to one or both of those questions led to inclusion of the patient in the progressive (active) GV group, whereas patients with no increase in lesion size or number were included in the stable GV group. Three hundred eighty-two patients were classified with progressive GV, whereas 135 patients were included in the stable GV group (Supplementary Table S1).

A total of 881 age matched unaffected individuals were included as controls in the study (Supplementary Table S1). Demographic data for patients and controls are shown in Supplementary Table S1. None of the healthy individuals or their relatives had any evidence of vitiligo and autoimmune disease. The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all subjects before performing the studies.

Genomic DNA preparation

Five milliliters venous blood was collected from the patients and unaffected subjects in K$_3$EDTA coated vacutainers (BD). Genomic DNA was extracted from whole blood using "QIAamp DNA Blood Kit" (Qiagen, Inc.) according to the manufacturer's instructions.

Genotyping of +874A/T SNP of IFNG

IFNG +874A/T genotyping was done using amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) method. DNA was amplified in 2 different PCRs with a generic antisense primer and one of the 2 allele specific sense primers (Supplementary Table S2). 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*) (Supplementary Table S2). 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$_2$O, 1.5 μL 10×PCR buffer, 0.3 μL 8 mM dNTPs (Sigma Chemical Co.), 0.3 μL of 10 μM allele-specific, and common primers (MWG Biotech), 0.3 μL of 2 μM control primers (*HGH*), 1.5 μL Mg^{2+} and 0.6 μL (5 U/μL) Taq Polymerase (Bangalore Genei). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc.) according to the protocol: 95°C for 1 min followed by 10 cycles of 95°C for 15 s, 62°C for 50 s, and 72°C for 40 s, then 20 cycles of 95°C for 20 s, 56°C for 50 s, and 72°C for 50 s.

The amplified products were separated by electrophoresis on 2.0% agarose gels and visualized under a UV transilluminator with a 100 base pair DNA ladder (Bioron). Two amplicons were available for each sample (one each specific for A or T 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 2 researchers independently and also confirmed by DNA sequencing).

Genotyping of CA microsatellite (rs3138557) of IFNG

IFNG CA microsatellite (rs3138557) was genotyped by High Resolution Melt (HRM) curve analysis using Light-Cycler® 480 Real-Time PCR protocol. The primers used for the genotyping are mentioned in Supplementary Table S2. Real-time PCR was performed in 20 μL volume using LightCycler 480 HRM Master (Roche Diagnostics GmbH) following the manufacturer's instructions. 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, 56°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 HRM 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.

Determination of IFNG, ICAM1 and GAPDH mRNA expression

RNA extraction and cDNA synthesis. Total RNA from whole blood was isolated and purified using Ribopure™-blood Kit (Ambion, Inc.) 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. cDNA synthesis was performed using 1 μg of total RNA by Thermo Scientific Verso™ cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Real-time PCR. The expression of *IFNG*, *ICAM1*, and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins) as shown in Supplementary Table S2. 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) following the manufacturer's instructions. The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 10 s, 65°C for 15 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase, a melt curve analysis was carried out to check the specificity of the products formed (Supplementary Fig. S1). The PCR cycle at which PCR amplification begins its exponential phase and product fluorescence intensity finally rises above background and becomes visible was considered as the crossing point (C_P) or cycle threshold (C_T). The ΔC_T or ΔC_P value was determined as the difference between the cycle threshold of target gene (*IFNG/ICAM1*) and reference gene (*GAPDH*). The difference between the 2 ΔC_P values (ΔC_P Controls and ΔC_P patients) was considered as $\Delta\Delta C_P$ to obtain the value of fold change ($2^{-\Delta\Delta C_P}$).

Estimation of serum IFN-γ levels

Serum levels of IFN-γ in patients with GV and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the Immunotech Human IFN-γ ELISA kit (Immunotech SAS) as per the manufacturer's protocol.

Statistical analyses

Evaluation of the Hardy–Weinberg equilibrium (HWE) was performed 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.). *P* values less than 0.01 were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio with respective confidence interval (95% CI) for disease susceptibility was also calculated. Age of onset analysis, relative gene expression of *IFNG*, and serum IFN-γ levels in patient and control groups were plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad Software, Inc.).

TABLE 1. DISTRIBUTION OF GENOTYPES AND ALLELE FREQUENCIES FOR *IFNG* +874A/T (RS2430561) INTRON 1 POLYMORPHISM IN GENERALIZED VITILIGO PATIENTS AND CONTROLS

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

Values are significant at $P \leq 0.01$ due to Bonferroni's correction.
'*n*' represents number of patients/controls.
[a]Vitiligo Patients versus controls using chi-squared test with 3×2 contingency table.
[b]Vitiligo Patients versus controls using chi-squared test with 2×2 contingency table.
GV, generalized vitiligo; HWE, Hardy–Weinberg equilibrium; CI, confidence interval; Ps, patients; C, controls.

TABLE 2. DISTRIBUTION OF GENOTYPES AND ALLELE FREQUENCIES FOR *IFNG* +874A/T (RS2430561) INTRON 1 POLYMORPHISM IN ACTIVE AND STABLE GENERALIZED VITILIGO PATIENTS

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

Values are significant at $P \leq 0.01$ due to Bonferroni's correction.
'*n*' represents number of patients.
[a]Active patients versus stable patients using chi-squared test with 3×2 contingency table.
[b]Active patients versus stable patients using chi-squared test with 2×2 contingency table.
AV, active vitiligo patients; SV, stable vitiligo patients.

Results

Analysis of association between intron 1 +874A/T (rs2430561) polymorphism of IFNG and susceptibility to GV

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 (Supplementary Fig. S2).

Intron 1 +874A/T polymorphism of *IFNG* gene was not found to be associated with GV patients ($P=0.485$) when genotypes were compared with chi-squared test-3×2 contingency table (Table 1). Also, there was no significant difference in allele frequencies of this polymorphism between patients and controls when compared with 2×2 contingency table ($P=0.274$) (Table 1). Both patients and control population were found to be in HWE for this polymorphism ($P=0.942$ and $P=0.847$, respectively) (Table 1). Interestingly, the frequency of minor 'T' allele was significantly higher in active cases as compared to stable cases of GV (45% versus 31%; $P=0.0003$) (Table 2). In addition, genotype frequencies

and allele frequencies for this polymorphism were also analyzed between male and female patients with GV and controls, which revealed no significant difference as detected by chi-squared test ($P=0.459$ and $P=0.030$, respectively) (Table 3).

Analysis of association between intron 1 CA microsatellite (rs3138557) polymorphism of IFNG and susceptibility to GV

HRM curve analysis for CA microsatellite (rs3138557) polymorphism was performed using LightCycler 480 Real-Time PCR. The 5 different CA repeats identified were, 12–12 CA, 13–13 CA, 12–13 CA, 12–14 CA, and 13–14 CA, based on normalized and temperature shifted difference plots followed by DNA sequencing of samples from each representative group (Supplementary Figs. S3 and S4).

The distribution of the *IFNG* microsatellite polymorphism (CA) was different between cases and controls ($P<0.001$). The genotype (CA) 12–12, that is, the homozygous state for allele 2 was significantly more frequent ($P=0.005$) in GV 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 4). The allelic frequency differed significantly between cases and controls ($P=0.004$). In GV patients, the allele 12 was significantly

TABLE 3. ASSOCIATION STUDIES FOR *IFNG* +874A/T (RS2430561) INTRON 1 POLYMORPHISM IN MALE AND FEMALE PATIENTS WITH GENERALIZED VITILIGO

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

Values are significant at $P \leq 0.01$ due to Bonferroni's correction.
'*n*' represents number of patients.
[a]Male patients versus female patients using chi-squared test with 3×2 contingency table.
[b]Male patients versus female patients using chi-squared test with 2×2 contingency table.
M, male patients; F, female patients.

TABLE 4. 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[a]	P value[b]
rs3138557 (CA Repeats)	Genotype	(n = 160)	(n = 210)		
	CA 12–12	92 (0.57)	48 (0.23)	< 0.001	0.005
	CA 13–13	32 (0.20)	49 (0.23)	< 0.001	NS
	CA 12–13	16 (0.10)	88 (0.42)	< 0.001	0.005
	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

Values are significant at $p \leq 0.01$ due to Bonferroni's correction.
[a]Comparison of the distribution of genotypes between the 2 groups.
[b]Comparison of the single genotype/allele against all the others.
NS, not significant.

($P = 0.004$) 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 4). The results suggest significant association of *IFNG* 12–12 CA repeats with GV susceptibility.

Effect of IFNG +874A/T polymorphisms on age of onset of GV

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) (Fig. 1A). Moreover, patients with genotype AT showed an early onset of the disease as compared to AA genotypes ($P = 0.011$) (Fig. 1A) suggesting the effect of the susceptible allele 'T' on the early onset of disease.

Interestingly, when male and female GV 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 ($P = 0.002$) (Fig. 1B).

Relative gene expression of IFNG in patients with GV and controls

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

Correlation of IFNG transcripts with +874A/T polymorphism

Further, the expression levels of *IFNG* were analyzed with respect to +874A/T (rs2430561) genotypes (Fig. 2C). 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 ($P = 0.057$).
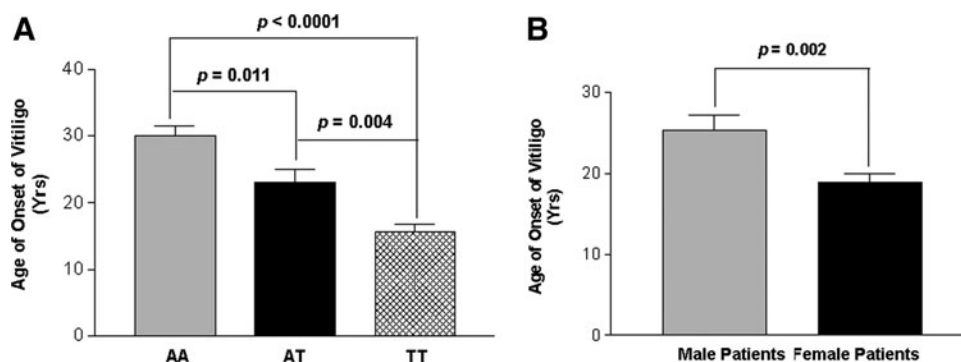

FIG. 1. Age of onset of the disease in generalized vitiligo (GV) patients. **(A)** Comparison of age of onset of the disease (years) with respect to *IFNG* A/T (rs2430561) polymorphism in 517 GV patients. Patients with TT genotype showed early age of onset of disease as compared to AA (mean age of onset ± SEM: 15.66 ± 1.145 versus 29.94 ± 1.592; $P < 0.0001$) and AT genotypes (mean age of onset ± SEM: 15.66 ± 1.145 versus 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 versus 29.94 ± 1.592; $P = 0.011$). **(B)** Comparison of age of onset of the disease (years) with respect to gender differences in 222 male patients and 295 female patients. Female patients showed early age of onset of disease as compared to male patients (Mean age of onset ± SEM: 18.87 ± 1.128 versus 25.32 ± 1.888; $P = 0.002$).
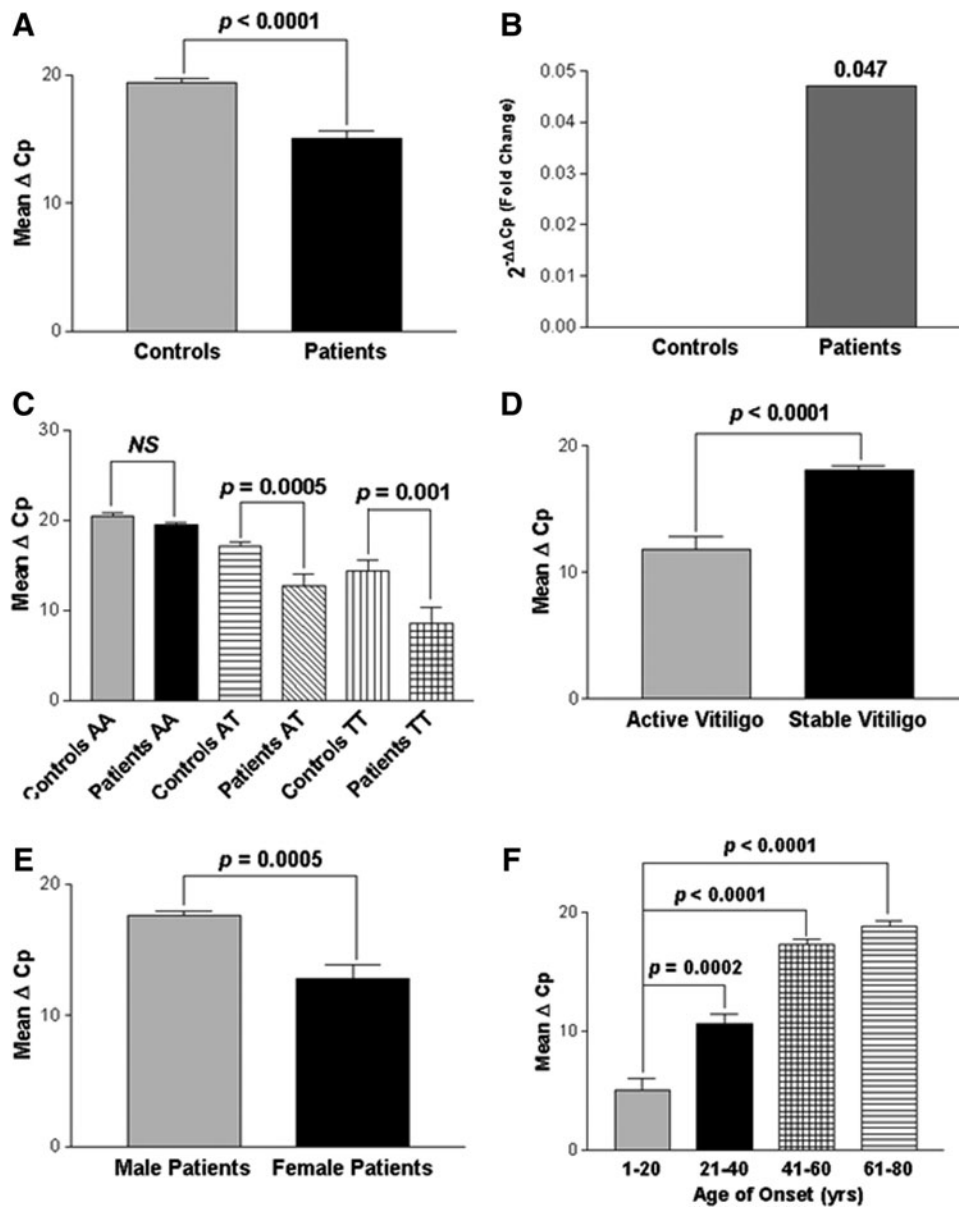
FIG. 2. Relative gene expression of *IFNG* in controls and GV patients. **(A)** Expression of *IFNG* transcripts in 175 controls and 122 GV patients as suggested by mean ΔCp. Patients showed significantly increased mRNA levels of *IFNG* as compared to controls (Mean ΔCp±SEM: 15.04±0.6603 versus 19.44±0.2806; $P < 0.0001$). **(B)** Expression fold change of *IFNG* transcripts in 166 GV patients against 175 controls showed 0.047-fold change as determined by $2^{-\Delta\Delta C_P}$ method. **(C)** Expression of *IFNG* transcripts with respect to *IFNG* +874A/T (rs2430561) polymorphism in 122 GV patients and 175 controls, as suggested by MeanΔCp. Patients showed significantly increased mRNA levels of *IFNG* with TT (MeanΔCp±SEM: 8.531±1.778 versus 14.40±1.242; $P = 0.001$) and AT (MeanΔCp±SEM: 12.72±1.317 versus 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 (MeanΔCp±SEM: 19.50±0.2929 versus 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 GV and 31 patients with stable GV, as suggested by MeanΔCp. Active GV patients showed significantly increased mRNA levels of *IFNG* as compared to stable GV patients (MeanΔCp±SEM: 11.79±1.034 versus 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, as suggested by MeanΔCp. Female patients showed significantly increased mRNA levels of *IFNG* as compared to male patients (MeanΔCp±SEM: 12.83±1.008 versus 17.63±0.3478; $P = 0.0005$). **(F)** Expression of *IFNG* transcripts with respect to different age groups in 122 GV patients, as suggested by MeanΔCp. Patients with the age of onset 1–20 years showed significantly increased expression of *IFNG* mRNA as compared to the age groups 21–40 years (MeanΔCp±SEM: 5.047±0.9394 versus 10.65±0.8343; $P = 0.0002$), 41–60 (MeanΔCp±SEM: 5.047±0.9394 versus 17.27±0.5239; $P < 0.0001$) and 61–80 years (MeanΔCp±SEM: 5.047±0.9394 versus 18.87±0.4213; $P < 0.0001$).
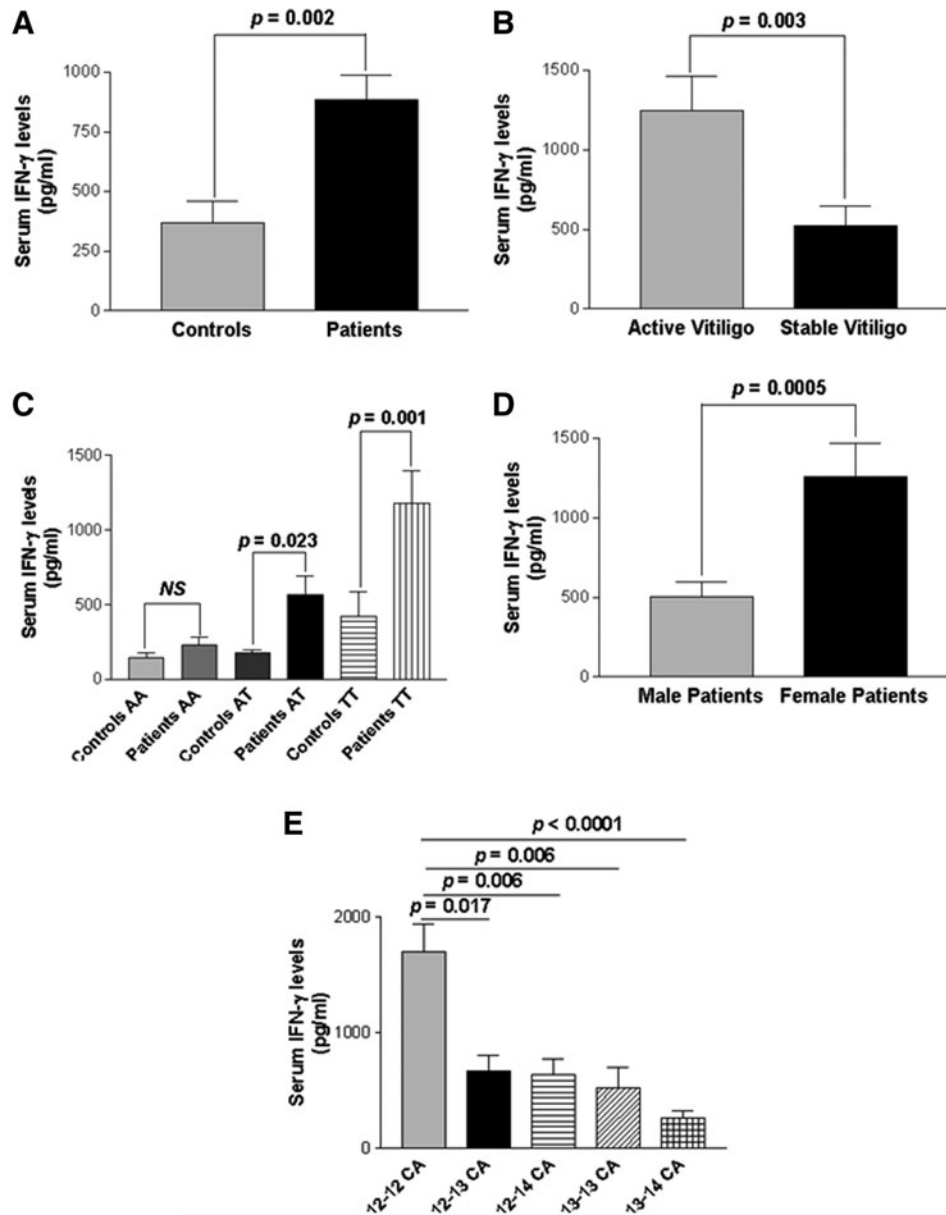
FIG. 3. Serum interferon-γ (IFN-γ) levels in controls and GV patients. **(A)** Serum IFN-γ levels in 236 controls and 214 GV patients. Patients showed significantly increased levels of IFN-γ as compared to controls (mean±SEM: 885.8±103.7 versus 367.1±90.53; $P = 0.002$). **(B)** Serum IFN-γ levels with respect to progression of the disease in 156 patients with active GV and 58 patients with stable GV. Active GV patients showed significantly increased levels of IFN-γ as compared to stable GV patients (mean±SEM: 1245±216.8 versus 523.1±121.4; $P = 0.003$). **(C)** Serum IFN-γ levels with respect to *IFNG* +874A/T (rs2430561) polymorphism in 214 GV patients and 236 controls. Patients showed significantly increased levels of IFN-γ with TT (mean±SEM: 1174±217.4 versus 421.4±161.1; $P = 0.001$) and AT (mean±SEM: 566.0±124.1 versus 175.2±21.85; $P = 0.023$) genotypes as compared to controls. No significant difference in IFN-γ levels was observed in patients with AA genotypes (mean±SEM: 229.4±48.73 versus 144.6±30.41; $P = 0.521$) as compared to controls. **(D)** Serum IFN-γ levels with respect to gender differences in 99 male patients and 137 female patients. Female patients showed significantly increased levels of IFN-γ as compared to male patients (mean±SEM: 1260±208.7 versus 503.5±96.47; $P = 0.0005$). **(E)** A total of 370 individuals were analyzed for serum IFN-γ levels with respect to *IFNG* CA microsatellite polymorphism. Individuals with 12–12 CA repeats showed significantly increased IFN-γ levels as compared to 12–13 CA (mean±SEM: 1424±252.2 versus 767.2±135.7; $P = 0.017$), 12–14 CA (mean±SEM: 1424±252.2 versus 632.9±137.5; $P = 0.006$), 13–13 CA (mean±SEM: 1424±252.2 versus 516.8±178.9; $P = 0.006$), and 13–14 CA (mean±SEM: 1424±252.2 versus 262.9±58.01; $P < 0.0001$) repeats.

Effect of IFNG expression on disease progression

In addition, we also checked the effect of *IFNG* expression on progression of the disease, that is, active and stable cases (Fig. 2D). Interestingly, active GV patients showed significant increase in expression of *IFNG* transcripts as compared

to the patients with stable GV ($P < 0.0001$) suggesting the involvement of IFN-γ in disease progression. Moreover, the susceptibility of the disease was also checked based on the gender differences and we found that female patients showed significantly higher *IFNG* expression as compared to male patients ($P = 0.0005$) (Fig. 2E).

When *IFNG* expression was monitored in different age of onset groups, patients with age of onset group: 1–20 years showed significantly increased expression of *IFNG* transcripts as compared to the age of onset groups: 21–40, 41–60, and 61–80 years ($P = 0.0002$, $P < 0.0001$, and $P < 0.0001$, respectively), suggesting the importance of IFN-γ in early onset of the disease (Fig. 2F).

Functional correlation of IFNG +874A/T and CA microsatellite (rs3138557) polymorphisms with its levels in the serum

To find any functional correlation of the investigated *IFNG* promoter polymorphisms with its level in the serum, IFN-γ levels were measured in 214 GV patients and 236 unaffected controls. GV patients showed significant increased serum IFN-γ levels as compared to controls ($P = 0.002$) (Fig. 3A). Moreover, when the patient groups were analyzed based on disease progression with respect to IFN-γ levels, patients with active GV had significantly higher IFN-γ levels as compared to stable GV ($P = 0.003$) (Fig. 3B).

Furthermore, when +874A/T genotypes were analyzed with IFN-γ levels, patients with TT and AT genotypes showed significantly higher levels of IFN-γ as compared to those of controls ($P = 0.001$, $P = 0.023$) (Fig. 3C). However, patients with AA genotype showed no significant difference in IFN-γ levels as compared to those of controls ($P = 0.521$). Additionally, when the male and female patients were analyzed with respect to IFN-γ levels, female patients showed significantly higher levels of IFN-γ as compared to male patients ($P = 0.0005$) (Fig. 3D).

Further, the 5 different CA repeats of *IFNG* were correlated with serum IFN-γ levels and interestingly, the 12–12 CA repeat showed significantly higher levels of IFN-γ as compared to longer CA repeats, that is, 12–13, 12–14, 13–13, and 13–14 ($P = 0.017$, $P = 0.006$, $P = 0.006$ and $P < 0.0001$) (Fig. 3E) suggesting the positive correlation of *IFNG* 12–12 CA repeats with higher levels of IFN-γ.

Relative gene expression of ICAM1 in patients with GV and controls

Comparison of the findings showed significant increase in expression of *ICAM1* transcripts in 122 GV patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values ($P = 0.008$) (Fig. 4A). The $2^{-\Delta\Delta C_P}$ analysis showed ∼0.369-fold change in the expression of *ICAM1* transcript in patients as compared to controls (Fig. 4B).

In addition, the effect of *ICAM1* expression on progression of the disease, that is, active and stable cases (Fig. 4C) revealed that active GV patients had significantly increased expression of *ICAM1* transcripts as compared to patients with stable GV, ($P = 0.008$) suggesting the involvement of *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 GV showed significantly higher *ICAM1* expression as compared to male patients ($P = 0.006$) (Fig. 4D). When *ICAM1* expression was monitored in different age of onset groups of patients, patients with age of onset group: 1–20 years showed significantly increased expression of *ICAM1* transcripts as compared to the age of onset groups: 21–40, 41–60, and 61–

80 years ($P = 0.0002$, $P < 0.0001$ and $P < 0.0001$, respectively) suggesting the importance of *ICAM1* in early onset of the disease (Fig. 4E).

Discussion

Vitiligo cannot be explained by simple Mendelian genetics; however, it is characterized by incomplete penetrance, multiple susceptibility loci, and genetic heterogeneity (Zhang and others 2005). We have earlier reported that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil and others 2006a). The present study also shows that 12.57% of GV patients have one or more first degree relative affected, suggesting the involvement of genetic factors in pathogenesis of GV. Recently, we have shown positive association of *HLA-A*33:01*, *HLA-B*44:03*, and *HLA-DRB1*07:01* with vitiligo patients from North India and Gujarat suggesting an autoimmune link of vitiligo in these cohorts (Singh and others 2012). We also showed that the 3 most significant class II region SNPs: rs3096691 (just upstream of *NOTCH4*), rs3129859 (just upstream of *HLA-DRA*), and rs482044 (between *HLA-DRB1* and *HLA-DQA1*) are associated with GV (Birlea and others 2013). The genotype-phenotype correlation of *CTLA4*, *IL-4*, and *TNFA* MYG1 polymorphisms supported the autoimmune pathogenesis of vitiligo in Gujarat population (Dwivedi and others 2011; Imran and others 2012; Laddha and others 2012; Dwivedi and others 2013a), whereas our earlier studies on *CAT*, *GPX*, *MBL2*, *ACE*, *PTPN22* polymorphisms did not show significant association (Shajil and others 2007; Dwivedi and others 2008, 2009; Laddha and others 2008).

Autoimmunity has been suggested to play a major role in the pathogenesis of GV (Nordlund and others 2006). The autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity (Kemp and others 2001; Shajil and others 2006b; Laddha and others 2013). Melanocyte-specific circulating autoantibodies (Cui and Bystryn 1995; Kemp and others 1997), autoreactive CD8^{+} cytotoxic T-cells and macrophages (Le Gal and others 2001; Mandelcorn-Monson and others 2003) that recognize pigment cell antigens have been detected in the sera of a significant proportion of vitiligo patients. In particular, active cases of vitiligo were demonstrated to have higher levels of autoantibodies and cytotoxic T-cells (Le Poole and Luiten 2008). Our recent study has also demonstrated circulating autoantibodies in the sera of 75% of Gujarat vitiligo patients as compared to unaffected individuals (Unpublished data). The autoimmune hypothesis gains further support from immunotherapy studies of melanoma patients (Rosenberg 1997). Twenty-six percent of melanoma patients responded to IL-2 based immunotherapy, developed vitiligo suggesting that antimelanotic T-cells which might be responsible for melanoma regression may also be linked to the destruction of normal melanocytes in vitiligo (Zeff and others 1997). Moreover, *in vitro* direct analysis of vitiliginous skin margins showed the presence of polarized CD4^{+} and CD8^{+} T-cells, which predominantly secrete IFN-γ and TNF-α might be associated with the destruction of melanocytes during active disease (Wańkowicz-Kalińska and others 2003). Our recent study has also shown dramatic increase in CD8^{+} T-cell number and significant decrease in Tregs number in circulation of active GV patients (Dwivedi and others 2013b).

Transcript and protein levels of cytokines depend on both genetic and environmental factors (Gottenberg and others
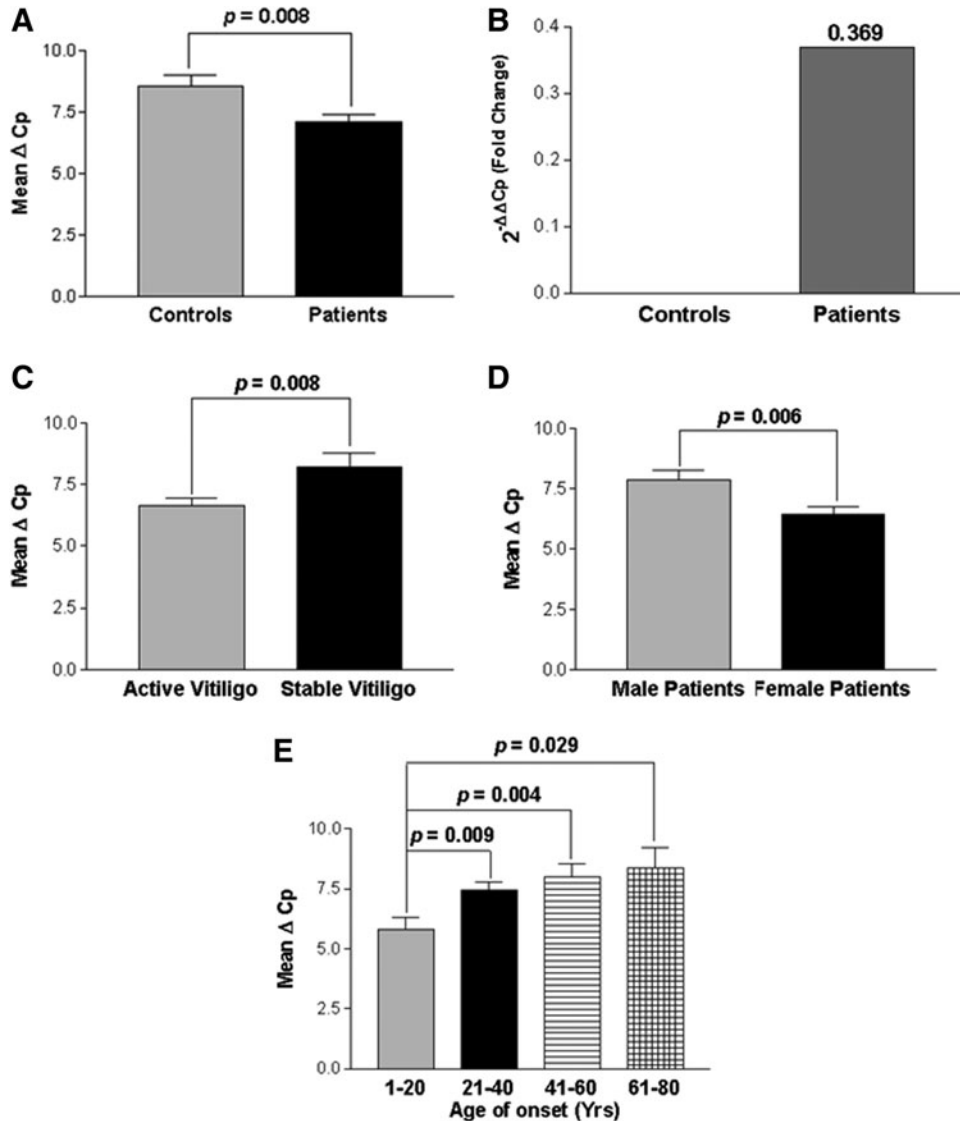
FIG. 4. Relative gene expression of intercellular adhesion molecule-1 (*ICAM1*) in controls and GV patients. **(A)** Expression of *ICAM1* transcripts in 175 controls and 122 GV patients, as suggested by MeanΔCp. Patients showed significantly increased mRNA levels of *ICAM1* as compared to controls (MeanΔCp±SEM: 8.552±0.4568 versus 7.112±0.2705; *P*=0.008). **(B)** Expression fold change of *ICAM1* transcripts in 122 GV 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 GV and 31 patients with stable GV, as suggested by MeanΔCp. Active GV patients showed significantly increased mRNA levels of *ICAM1* as compared to stable GV patients (MeanΔCp±SEM: 6.662±0.2852 versus 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, as suggested by MeanΔCp. Female patients showed significantly increased mRNA levels of *ICAM1* as compared to male patients (MeanΔCp±SEM: 7.890±0.4093 versus 6.435±0.3266; *P*=0.006). **(E)** Expression of *ICAM1* transcripts with respect to different age groups in 122 GV patients, as suggested by MeanΔCp. Patients with the age of onset 1–20 years showed significantly increased expression of *ICAM1* mRNA as compared to the age groups 21–40 years (MeanΔCp±SEM: 5.819±0.4737 versus 7.436±0.3662; *P*=0.009), 41–60 (MeanΔCp±SEM: 5.819±0.4737 versus 7.985±0.5435; *P*=0.004) and 61–80 years (MeanΔCp±SEM: 5.819±0.4737 versus 8.384±0.8328; *P*=0.029).

2004). Analysis of cytokines 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. Pravica and others (1999) reported that the *IFNG* +874A/T SNP directly affects the level of IFN-γ production and correlates with the presence of the 'A' allele and low production of IFN-γ as compared to 'T' allele. Further, the study suggested that this

polymorphism ('T' allele) coincided with a putative NF-κB binding site that could have functional consequences for transcription of the human *IFNG* gene, which could directly increase the level of IFN-γ production (Pravica and others 2000). The present study showed significant increase in *IFNG* mRNA and protein levels in GV patients as compared to controls. 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 (Moretti and others 2002a, 2002b; Grimes and others 2004). Recent studies have shown an increased *IFNG* mRNA levels in patient's involved skin (Grimes and others 2004; Schoenborn and Wilson 2007; Wang and others 2011; Reimann and others 2012) and also in PBMCs (Reimann and others 2012) compared to controls. Moreover, animal model studies for vitiligo have also confirmed the role of IFN-γ in vitiligo pathogenesis (Gregg and others 2010). Recently, increased expression of *IFNG* has been observed during early and active stages of Smyth line vitiligo (Shi and Erf 2012). These reports along with the present study emphasize the role of IFN-γ in the pathogenesis of GV.

Our results suggest that +874A/T intron1 polymorphism of *IFNG* gene is not associated with GV susceptibility in Gujarat population and our results are in accordance with those of Namian and others (2009). However, the genotype-phenotype correlation for +874A/T polymorphism suggested that *IFNG* mRNA and protein levels were higher in patients with homozygous TT and heterozygous AT genotypes compared with controls indicating the profound effect of 'T' allele with increased levels of IFN-γ in patients. Moreover, the 'T' allele frequency was significantly higher in patients with active GV as compared to stable GV indicating the role of +874 'T' allele in disease progression. In addition, active cases of GV showed increased mRNA and protein levels of IFN-γ as compared to stable cases, further confirming the important role of IFN-γ in disease progression. Age of onset analysis with *IFNG* +874A/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 years) had significantly higher levels of *IFNG* transcript as compared to those of late onset groups suggesting the crucial role of IFN-γ in early phase of the disease. Furthermore, a gender-based study indicated that female patients had an early onset of the disease and higher levels of *IFNG* mRNA and protein levels as compared to males suggesting that females have increased susceptibility towards GV, implicating gender biasness in the development of autoimmunity (Panchanathan and Choubey 2012).

IFN-γ levels have also been monitored in certain autoimmune diseases. Active cases of systemic lupus erythematosus (SLE) and mixed connective tissue disease showed increased levels of IFN-γ suggesting its role in the autoimmune disease pathogenesis (Funauchi and others 1991). Rekha and others (2006) suggested a differential association of high (T) and low (A) producing alleles of *IFNG* 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. In addition, a family based study (Schena and others 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 IFN-γ as compared to +874T allele (Schena and others 2006). Furthermore, the T allele of +874A/T polymorphism was found to be associated with elevated *IFNG* expression in Korean patients with SLE (Kim and others 2010).

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 and oth-

ers 2009). The study also confirms decreased serum IFN-γ levels along with the presence of homozygous AA genotypes. Further, the 2 North Indian studies reported significant association of *IFNG* +874A/T polymorphism with increased risk of cervical cancer (Kordi and others 2008; Gangwar and others 2009). Another study on South Indian population suggested a lack of functional association of *IFNG* +874A/T polymorphism with tuberculosis (Vidyarani and others 2006). These Indian cohort studies for *IFNG* +874A/T SNP association prompted us to investigate the role of *IFNG* in Gujarat population with GV.

The *IFNG* has a $(CA)_n$ repeat element within the first intron (Ruiz-Linares 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 (Agarwal and others 2000). It has been reported that good IFN-γ producers are considered to be individuals with 12 CA repeats and a 'T' at the polymorphic site (Pravica and others 1999, 2000). Individuals with the presence of 'A' allele with 13 repeats have low IFN-γ generation potential (Lee and others 2001; Miyake and others 2002). The current study reports for the first time significant association of 12 CA repeats with GV susceptibility. Interestingly, the serum IFN-γ 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 IFN-γ production. However, the CA microsatellite polymorphism has been associated with increased susceptibility to several autoimmune diseases, such as asthma (Nagarkatti and others 2002), type-1 diabetes (Jahromi and others 2000), polymyalgia rheumatica (PMR) (Gonzalez-Gay and others 2004), rheumatoid arthritis (Hanjani-Khani and others 2000) etc.

ICAM1, is important for activating T-cells and recruiting leukocytes (Hedley and others 1998). Beyond its classically described function as an adhesion and viral entry molecule, ICAM1 on the surface of T-cells is thought to participate in signal transduction affecting several T-cell functions, including activation, proliferation, cytotoxicity, and cytokine production and thereby, plays an important role in modulating autoimmune diseases (Stanciu and Djukanovic 1998). Furthermore, IFN-γ and TNF-α induce the expression of ICAM1 on the cell-surface of melanocytes (Yohn and others 1990). ICAM1 protein levels are upregulated in vitiligo skin and in melanocytes from perilesional vitiligo skin (Al Badri 1993). Increased expression of ICAM1 on the melanocytes enhances T cell- melanocyte attachment in the skin and may be involved in the destruction of melanocytes in vitiligo (Al Badri 1993). The present study also showed increased expression of *ICAM1* in GV patients suggesting that increased IFN-γ levels might be responsible for increased *ICAM1* expression in GV patients. Moreover, the *ICAM1* expression was increased in active cases of GV as compared to stable GV suggesting its role in progression of the disease. The *ICAM1* expression was increased at 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 GV.

In humans, IFN-γ is implicated in pathology of several autoimmune diseases, including SLE (Lee and others 2001), multiple sclerosis (Panitch and others 1987), allergic encephalomyelitis (Espejo and others 2001), autoimmune

nephritis (Heremans and others 1978), and type-1 diabetes (Jahromi and others 2000). IFN-γ- a Th1 cytokine is critical for both innate and adaptive immunity and the importance of IFN-γ in the immune system stems from its immunostimulatory and immunomodulatory effects. In addition, its effect on apoptosis has been suggested as a crucial role in the context of autoimmunity. IFN-γ enhances human B-cell proliferation (Francois and others 1988), activates antigen-presenting cells (APCs) and promotes Th1 differentiation and inhibits Th2 development thereby plays an important role in modulating autoimmune diseases (Oriss and others 1997). IFN-γ promotes specific cytotoxic immunity by indirect mechanisms, such as growth inhibition of Th2 populations and upregulation of antigen processing, presentation, and APC costimulatory molecules, thereby, increasing CD4$^+$ differentiation (Schroder and others 2004). It has been demonstrated that an imbalance between Th1 and Th2 cytokine production is highly correlated with the induction and development of several autoimmune diseases (Kidd 2003). Th1 responses, as characterized by IFN-γ, have been established in vitiligo (Sugita and others 2006). T-cells expanded from peri-lesional vitiligo skin show a predominately Type 1 cytokine profile (ie, IFN-γ and TNF-α) (Wań-kowicz-Kalińska and others 2003) and the treatment of vitiligo by using IFN-γ inhibitors has also shown positive therapeutic responses (Skurkovich and Skurkovich 2006); these reports suggest the crucial role of IFN-γ in pathogenesis of GV.

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 IFN-γ, TNF-α, or IL-1 released by lymphocytes and keratinocytes can initiate apoptosis (Huang and others 2002). Recently, we have also shown that increased TNF-α transcript and protein levels correlate with disease progression and higher susceptibility towards vitiligo (Laddha and others 2012). Since, IFN-γ has a role in inducing melanocyte apoptosis and destruction in GV, genetic association studies with *IFNG* polymorphisms in different ethnic populations need to be explored.

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

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Prof. Rasheedunnisa Begum
Department of Biochemistry
Faculty of Science
The Maharaja Sayajirao University of Baroda
Vadodara 390002
Gujarat
India

E-mail: rasheedunnisab@yahoo.co.in