List of Publications

Imran M*, **Laddha NC***, Dwivedi M, Mansuri MS, Singh J, Rani R, Gokhale RS, Sharma VK, Marfatia YS and Begum R (2012). Interleukin-4 genetic variants correlate with Its transcript and protein levels in vitiligo patients. ***Brit J Dermatol.*** 167: 314-323. (*Equal contribution)

Dwivedi M, **Laddha NC**, Imran M, Shah BJ and Begum R (2011). Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) in isolated vitiligo: a genotype-phenotype correlation. ***Pigment Cell Melanoma Res.*** 24: 737-740.

Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK, Gupta S, **Laddha NC**, Dwivedi M, Begum R, The Indian Genome Variation Consortium, Gokhale RS and Rani R (2012). HLA alleles and amino acid signatures of the peptide binding pockets of HLA molecules in Vitiligo. ***J Invest. Dermatol.*** 132: 124-134.

Dwivedi M, Gupta K, Gulla KC, **Laddha NC**, Hajela K and Begum R (2009). Lack of genetic association of promoter and structural variants of mannan-binding lectin (*MBL*-2) gene with susceptibility to generalized vitiligo. ***Brit J Dermatol.*** 161: 63-69.

Dwivedi M*, **Laddha NC***, Shajil EM, Shah BJ and Begum R (2008). The *ACE* gene I/D polymorphism is not associated with generalized vitiligo susceptibility in Gujarat population. ***Pigment Cell Melanoma Res.*** 21: 407-408. (*Equal contribution)

Laddha NC, Dwivedi M, Shajil EM, Prajapati H, Marfatia YS and Begum R (2008). Association of *PTPN*22 1858C/T polymorphism with vitiligo susceptibility in Gujarat population. ***J Dermatol Sci.*** 49: 260-262.

Shajil EM, **Laddha NC**, Chatterjee S, Gani AR , Malek RA , Shah BJ and Begum R (2007). Association of catalase T/C exon 9 and glutathione peroxidase codon

200 polymorphisms in relation to their activities and oxidative stress with vitiligo susceptibility in Gujarat population. ***Pigment Cell Res.*** 20: 405-407.

Manuscripts under Communication

Laddha NC, Dwivedi M, Mansuri MS, Ansarullah, Ramachandran AV, Dalai S and Begum R. Vitiligo: interplay between oxidative stress and immune system.

Laddha NC, Dwivedi M and Begum R. Increased Tumor Necrosis Factor (*TNF*)-α and its promoter polymorphisms correlate with disease progression and higher susceptibility towards vitiligo.

Dwivedi M, **Laddha NC**, Mansuri MS, Shah K, Begum R. Correlation of increased *MYG*1 expression and its promoter polymorphism with disease progression and higher susceptibility in vitiligo patients.

Birlea SA, Ahmad FJ, Uddin RM, Ahmad S, Begum R, **Laddha NC**, Dwivedi M, Mansuri MS, Jin Y, Gowan K, Riccardi SL, Ben S, Fain PR and Spritz RA. Association of Generalized Vitiligo with HLA Class II Loci in Patients from the Indian Subcontinent.

Oral and Poster Presentations

Laddha NC, Dwivedi M, M. Imran, Shah K, Shah BJ and Begum R. "Role of TNF-α promoter polymorphisms in vitiligo susceptibility: a genotype-phenotype correlation." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M.S. University of Baroda, Vadodara.

Dwivedi M, **Laddha NC**, M. Imran, Shah K, Shah BJ and Begum R. "Role of interferon-γ polymorphisms in vitiligo susceptibility." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M. S. University of Baroda, Vadodara.

Mansuri MS, **Laddha NC**, Dwivedi M, Imran M, Agarwal N, Vasan K, Marfatia YS and Begum R. "Association of glutathione peroxidase 1 structural polymorphisms in relation to its activity and oxidative stress with vitiligo susceptibility." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M. S. University of Baroda, Vadodara.

Agarwal N, **Laddha NC**, Dwivedi M, Imran M, Mansuri MS , Patel HH , Shah AM , Marfatia YS and Begum R "Genetic polymorphisms of Neuropeptide-Y and Interleukin-1 beta may confer susceptibility to vitiligo." **XXVI Gujarat Science Congress**, Feb 26th 2012 at The M.S. University of Baroda, Vadodara.

Rani R, Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK, Gupta S, **Laddha NC**, Dwivedi M, Begum R, Indian Genome Variation Consortium, Gokhale RS. "Functional implications of MHC associations in vitiligo." **37th Annual Meeting of The American Society for Histocompatibility and Immunogenetics (ASHI),** Sheraton New Orleans, October 17-21, 2011.

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Dwivedi M, **Laddha NC**, Imran M, Ichhaporia V and Begum R. "Association of Cytotoxic T Lypmhocyte Associated Antigen-4 (*CTLA*-4) 3' UTR CT60A/G and exon1 +49A/G single nucleotide polymorphisms with vitiligo susceptibility." 4th Pan Arab Human Genetics Conference on "Genomics of Human Diversity and Heritable Disorders" **Human Genome Meeting-2011**, 14th -17th March, 2011, Dubai.

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Imran M, **Laddha NC**, Shah AM, Panchal VN, Sinh MK, Parmar CB, Raimalani VM and Begum R. "Association of Interleukin-4 Intron 3 INDEL polymorphism with Vitiligo susceptibility in Gujarat population." **Oral presentation at National Conference on CME** in Immunology, The M. S. University of Baroda, November 27-28, 2009. **(Awarded the First Prize)**

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Gani AR, Dwivedi M, **Laddha NC**, Shajil EM and Begum R. "*PTPN22* Single Nucleotide Polymorphism and *ACE* I/D Polymorphism in Gujarat vitiligo patients." Oral presentation at Science Excellence-2008 (SCIXL-2008) the State level Paper Presentation Competition held on 5th January 2008 at Gujarat University, Ahmedabad.. **(Awarded the First Prize)**

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LETTER TO THE EDITOR

Association of catalase T/C exon 9 and glutathione peroxidase codon 200 polymorphisms in relation to their activities and oxidative stress with vitiligo susceptibility in Gujarat population

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Dear Sir,

Vitiligo is a common depigmenting disorder resulting from the loss of melanocytes in the skin and affects 1–2% of the world population. The incidence of vitiligo is found to be 0.5–2.5% in India. Gujarat and Rajasthan states have the highest prevalence i.e. ~8.8% (Shajil et al., 2006a,b,c). Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction. We have earlier reported an alteration in the systemic antioxidant system with a significant reduction in catalase (CAT) and glutathione peroxidase (GPX) activities in vitiligo patients (Agrawal et al., 2004; Shajil and Begum, 2006). Here, we report a case-control study for the well-documented *CAT* exon 9 T/C (rs769217) and *GPX*1 codon 200 proline to leucine (rs1050450) polymorphisms and their relation to activities and lipid peroxidation (LPO) levels in Gujarat vitiligo patients where the prevalence of vitiligo is very high.

DNA was isolated from blood of 126 vitiligo patients and 143 age-matched healthy controls. Oligonucleotide primers used for PCR amplification of *CAT* exon 9 were 5′-GCCGCCTTTTTGCCTATCCT-3′ (forward primer) and 5′-TCCCGCCCATCTGCTCCAC-3′ (reverse primer) and for *GPXI* exon 2 were 5′-TGTGCCCCTACGCAGGTACA-3′ (forward primer) and 5′-CCAAATGACAATGACACAGG-3′ (reverse primer). For Restriction Fragment Length Polymorphism (RFLP) analysis, *CAT* exon 9 amplicon was digested with *Bst*XI and subjected to 15% PAGE; *GPXI* exon 2 amplicon was digested with *Apa*I and subjected to 2% AGE. Erythrocyte LPO, CAT, and GPX activities in vitiligo patients and controls were assayed by the standard methods (Agrawal et al., 2004).

The results of RFLP analysis of T/C silent substitution in *CAT* exon 9 (Asp389) is shown in Table 1. The observed allele frequencies of C and T were 0.867 and 0.133, respectively, in controls; 0.822 and 0.178 in vitiligo patients (Table 1). The allele frequencies of this T/C Single Nucleotide Polymorphism (SNP) did not differ significantly between the control and patient population ($p < 0.434$). When the observed control and patient genotype frequencies were compared with the expected values using a 3×2 contingency table in a standard chi-squared test (Table 1) they did not show any significant change ($p = 0.269$) suggesting that there is no possible association of the T/C exon 9 (*Bst*XI) *CAT* marker with vitiligo. The observed genotype frequencies of the control population did not differ significantly from those predicted by Hardy–Weinberg equation ($p < 0.259$) Also the patient population did not deviate from Hardy–Weinberg equilibrium ($p < 0.194$). The results of RFLP analysis of C/T polymorphism in *GPX* 1 exon 2 (Pro → Leu) is shown in Table 1. The observed *GPX* 1 allele frequencies of C and T were 0.721 and 0.279, respectively, in controls; 0.742 and 0.258 in vitiligo patients (Table 1). The allele frequencies of this T/C SNP did not differ significantly between the control and patient population ($p < 0.313$). When the observed control and patient genotype frequencies were compared with the expected values using a 3×2 contingency table in a standard chi-squared test (Table 1) they did not show any significant change ($p = 0.599$) suggesting that there is no association of the C/T exon 2 (*Apa*1) *GPX* 1 marker with vitiligo. The observed genotype frequencies of the control population differed significantly from those predicted by Hardy–Weinberg equation ($p < 0.0350$). Also the patient population is deviated from Hardy–Weinberg equilibrium ($p < 0.0037$).

Catalase activity did not show any significant change in vitiligo patients compared with controls. Also, CAT activity did not differ significantly between patients with C/C and C/T genotypes. However, GPX activity was found to be significantly decreased ($p < 0.0001$) in vitiligo patients compared with controls. Also, GPX activity was significantly decreased ($p < 0.0486$) in patients with

Table 1. Distribution of genotypes and alleles for the T/C SNP in *CAT* exon 9 and *GPX 1* exon 2 in vitiligo patients and control population

		Observed genotype counts			Observed allele frequencies		Expected genotype counts			
	N	C/C	C/T	T/T	C	T	C/C	C/T	T/T	p-value
Catalase										
Controls	143	106	36	1	0.867	0.133	107.54	32.89	2.57	0.259
Patients	126	82	43	1	0.822	0.178	85.18	36.92	3.91	0.194
p-value	0.269									
Glutathione peroxidase										
Controls	143	68	70	5	0.721	0.279	74.36	57.49	11.15	0.0350
Patients	126	63	61	2	0.742	0.258	69.3	48.26	8.44	0.0037
p-value	0.599									

C/C genotype compared with C/T genotype. LPO levels showed significant increase ($p < 0.0008$) in vitiligo patients compared with controls, which is an index for oxidative stress. However, LPO levels did not differ significantly between patients with *GPX* 1 and *CAT* C/C and C/T genotypes.

Casp et al. (2002) and Gavalas et al. (2006) showed an association between vitiligo susceptibility and *CAT* T/C SNP at codon 389 in Caucasian population and English population respectively. However, in Korean population the T/C *CAT* exon 9 genotype distribution and allele frequencies were not significantly associated with vitiligo (Park et al., 2006), which is in accordance with our study. Thus it seems that the genotype distribution of *CAT* T/C is different in different ethnic groups. Our results vary from the Caucasian and English population may be because of the racial differences. This study also shows that there is no change in the CAT activity in vitiligo patients compared with controls and our results are in accordance with our previous studies (Agrawal et al., 2004; Amina et al., 2006). However, there are reports showing significant reduction in CAT activity in vitiligo patients compared with controls (Passi et al., 1998). Also patients with CC and CT genotypes have not shown any significant change in the CAT activity. The role of *GPX* 1 polymorphism is not addressed so far in vitiligo but based on our previous results (Agrawal et al., 2004) we have made an attempt to study its association with vitiligo. Nevertheless, Ratnasinghe et al. (2000) showed that the *GPX* 1 codon 200 polymorphism was associated with increased risk of lung cancer in Finnish population. It was also reported that leucine containing *GPX* 1 allele was more frequently associated with breast cancer than the proline containing allele (Hu and Diamond, 2003). Conversely, our study could not find any association of this polymorphism with vitiligo, however the proline containing *GPX*1 allele (C/C) showed significant reduction ($p < 0.0486$) in GPX activity in vitiligo patients compared with patients with C/T genotype. Surprisingly, this change in activity was not observed in C/C and C/T genotypes of control population. In addition, allelic variants are reported in the *GPX 1* gene with low levels of GPX activity (Hu and Diamond, 2003; Ratnasinghe et al., 2000). Thus our results suggest that other SNPs in vitiligo patients could be contributing for the reduced GPX activity. Our results show a significant increase in LPO levels in vitiligo patients compared with controls. In patients *CAT* C/C genotype showed higher LPO levels compared with C/T genotype. However, no change is observed in LPO levels in vitiligo patients with different genotypes of *GPX* 1.

The present study shows that the well-documented *CAT* exon 9 T/C and *GPX* 1 codon 200 polymorphisms may not be associated with Gujarat vitiligo patients where the prevalence of vitiligo is alarmingly high. These results suggest for the presence of novel SNPs in Gujarat vitiligo population. Ours is the first report on *CAT* and *GPX* 1 polymorphisms in Gujarat vitiligo patients.

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LETTER TO THE EDITOR

Association of *PTPN22* 1858C/T polymorphism with vitiligo susceptibility in Gujarat population

Sir,

Vitiligo is a depigmenting disorder resulting from the loss of functional melanocytes in the skin and affects 1–2% of the world population. Gujarat state has the highest prevalence, i.e., ~8.8%. Though vitiligo is extensively addressed in the past five decades, its etiology is still being debated [1]. Our biochemical studies have shown an impaired antioxidant defense mechanism in Gujarat vitiligo patients compared to controls [2,3]. Vitiligo is hypothesized to be of autoimmune origin [4]. Protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene C/T single nucleotide polymorphism at 1858 (rs2476601) position was reported to be associated with increased risk of autoimmune diseases including generalized vitiligo [5]. The aim of this study was to find out whether well-documented *PTPN22* 1858C/T polymorphism is associated with Gujarat vitiligo patients.

DNA was isolated from blood of 126 generalized vitiligo patients and 140 age-matched healthy controls. Oligonucleotide primers used for PCR amplification were 5′-GCCTCAATGAACTCCTCAA-3′ (forward primer) and 5′-CCTCCTGGGTTTGTACCTTA-3′ (reverse primer). Amplified PCR product (400 bp) was confirmed by agarose gel electrophoresis with 100 bp DNA ladder (MBI Fermentas).

For restriction fragment length polymorphism (RFLP) analysis, PCR-amplified products were digested with *Xcm*I (New England Biolabs). The amplicon with homozygous T/T allele of *PTPN22* is cleaved by *Xcm*I yielding 238 and162 bp fragments, whereas the amplicon with homozygous C/C allele remains uncut yielding 400 bp band, while the amplicon with heterozygous C/T allele yields three fragments of 400, 238 and 162 bp length (Fig. 1). The digested products were separated on 2% agarose gel along with 100 bp DNA ladder. Ethidium bromide stained gels were visualized under UV light.

The results of RFLP analysis of *PTPN22* 1858C/T polymorphism is shown in Table 1. The observed *PTPN22* allele frequencies of C and Twere 0.978 and 0.022, respectively, in controls; and 0.992 and 0.008, respectively, in vitiligo patients (Table 1). The allele frequencies of this C/T SNP did not differ significantly between the control and patient population ($p < 0.560$). When the observed control and patient genotype frequencies were compared with the expected values using a 3 × 2 contingency table in a standard χ^2-test (Table 1) they did not show any significant change ($p = 0.198$), suggesting that there is no association of the C/T (*Xcm*I) *PTPN22* marker with vitiligo. The observed genotype frequencies of the control population did not differ significantly from those predicted by Hardy Weinberg equation ($p < 0.932$). Also the patient population is not deviated from Hardy Weinberg equilibrium ($p < 0.994$).

PTPN22 gene is known to play an important role in negative T cell regulation. The 1858C/T polymorphism of *PTPN22* has been well documented for its association with several autoimmune diseases. However, our study shows that there is no association

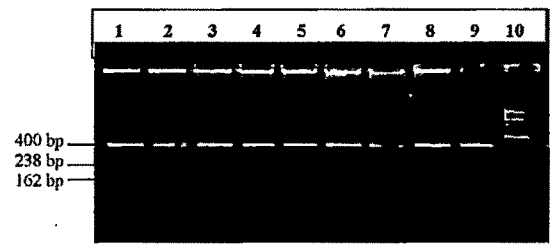

Fig. 1 PCR–RFLP analysis of *PTPN22* 1858C/T SNP: 2% agarose gel electrophoresis after overnight digestion of the PCR product with *Xcm*I. Lanes 1, 3, 4, 5, 6, 8, and 9 show subjects with homozygous alleles (C/C) with one intact band. Lanes 2 and 7 show subjects with heterozygous alleles (C/T) showing digestion of the 400 bp product into 238 bp and 162 bp bands. No subject with homozygous allele (T/T) was observed. Lane 10 shows a 100-bp ladder.

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Table 1 Distribution of genotypes and alleles for the 1858C/T SNP in *PTPN22* in vitiligo patients and control population.

	N	Observed genotype count			Observed allele frequencies		Expected genotype count			*p*-value
		C/C	C/T	T/T	C	T	C/C	C/T	T/T	
Controls	140	134	6	0	0.978	0.022	133.84	6.02	0.14	0.932
Patients	126	124	2	0	0.992	0.008	123.98	2.02	0.013	0.994
p-value						0.198				

between *PTPN22* 1858C/T polymorphism and vitiligo in Gujarat population. Canton et al. [5] have shown an association of *PTPN22* 1858C/T polymorphism with generalized vitiligo in Caucasian population. No other association studies are available on *PTPN22* 1858C/T polymorphism and vitiligo. There are several reports suggesting an association of 1858C/T polymorphism with other autoimmune diseases [6,7]. On the contrary, several reports also suggest a non-association of *PTPN22* 1858C/T polymorphism with autoimmune diseases [7–9]. These contradictory reports may be because of the differences in ethnicity of the studied populations. Ray et al. [8] have reported a non-association of *PTPN22* 1858C/T polymorphism with sporadic idiopathic hypoparathyroidism in Indian population.

PTPN22 gene encodes a lymphoid-specific phosphatase (Lyp). Lyp is an intracellular protein tyrosine phosphatase (PTP) and is physically bound to the SH3 domain of the Csk kinase through proline-rich motif and acts as an important suppressor of kinases that mediate T-cell activation. The ability of Csk and Lyp to inhibit T-cell receptor signaling requires their physical association. The C1858T substitution changes the amino acid from arginine (620R) to tryptophan (620W) at codon 620. This residue resides in the P1 proline-rich motif that is involved in binding to the SH3 domain of Csk. *In vitro* experiments have shown that the T-allele of *PTPN22* binds less efficiently to Csk than the C-allele does, suggesting that T-cells expressing the T-allele may be hyper-responsive, and consequently, individuals carrying this allele may be prone to autoimmunity [6,7].

The exact genetic mechanism of autoimmune diseases is not yet well understood. Autoimmune diseases are also influenced by environmental factors in addition to genetic factors. The study could not attain statistical significance consequent to lower prevalence rate of 1858T allele in the studied population. Interestingly, 1858T allele has been found to be absent in East Asians, including Japanese [9], Chinese [7], and South Asian Indians [8].

The present study shows that *PTPN22* 1858C/T polymorphism may not be associated with Gujarat vitiligo patients. Our studies on catalase exon 9 T/C and glutathione peroxidase codon 200 C/T polymorphisms also have not shown any significant association with vitiligo [10]. These results suggest for the presence of novel SNPs in Gujarat vitiligo population. This is the first report on *PTPN22* 1858C/T polymorphism in Gujarat vitiligo patients where the prevalence of vitiligo is alarmingly high.

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LETTER TO THE EDITOR

The *ACE* gene I/D polymorphism is not associated with generalized vitiligo susceptibility in Gujarat population

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Dear Sir,

Vitiligo is a hypomelanotic disease characterized by circumscribed depigmented macules and affects 0.5–2% of the world population (Passeron and Ortonne, 2005). However, in India Gujarat has the highest prevalence i.e., approximately 8.8% (Valia and Dutta, 1996). Vitiligo is hypothesized to be of autoimmune origin and genetic factors may also involve in precipitating this disease. Our earlier study suggests that 13.7% of Gujarat vitiliginous patients have at least one first-degree relative affected (Shajil et al., 2006). Angiotensin-converting enzyme (ACE), a key enzyme in the renin–angiotensin system, plays an important role in the physiology of the vasculature, blood pressure and inflammation. *ACE* has also been found to be associated with several autoimmune disorders (Scholzen et al., 2003; Vuk-Pavlovic and Rohrbach, 1988). The *ACE* gene is located on chromosome 17q23.3 and an insertion/deletion (I/D) polymorphism of a 287-base pair repetitive sequence in intron 16 of the *ACE* gene (NCBI: AF118569; repeat region 14094–14381) is reported to be associated with the development of vitiligo (Jin et al., 2004). The I/D polymorphism of the *ACE* gene accounts for the variability of serum ACE activity, D/D genotype having the highest and I/I genotype having the lowest ACE activity (Rigat et al., 1990). The aim of this study was to investigate the distribution of *ACE* I/D genotypes in generalized vitiligo patients and in healthy controls of Gujarat population to find the relationship between *ACE* I/D polymorphism and vitiligo.

A total of 125 generalized vitiligo patients and 156 ethnically matched healthy controls of Gujarat population were examined for *ACE* I/D polymorphism. The patients selected for this study had no other associated diseases. The DNA was prepared from blood samples after written informed consent was obtained. Oligonucleotide primers used for *ACE* intron 16 PCR amplification were 5′-CTGGAGACCACTCCCATCCTTTCT 3′-(forward primer) and 5′-GATGTGGCCATCACATTCGTCAGAT 3′ (reverse primer).

PCR amplification was performed in 25 μl reaction system containing 50 ng of genomic DNA and 20 pmol of each primer under the following conditions: 94°C for 10 min, 94°C for 30 s, 58°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. Amplified PCR products were analyzed on 2% agarose gel electrophoresis with 100 bp DNA ladder to identify the three genotype patterns: I/I (a 480 bp fragment), D/D (a 193 bp fragment) and I/D (two fragments, i.e., 480 and 193 bp). The distribution of the *ACE* I/D genotypes for patients and control subjects were compared by using the chi-square test with 2×2 and 3×2 contingency tables and P-values less than 0.05 were considered statistically significant. The statistical power of the current study was determined by using the G * Power software (Faul et al., 2007).

The observed allele and genotype frequencies for the *ACE* I/D polymorphism in controls and patient population are shown in Table 1. No significant difference in the genotype frequencies of I/I, I/D and D/D genotypes was observed between vitiligo patients and control subjects ($P = 0.459$) (Table 1), suggesting that there is no association of the *ACE* I/D polymorphism with vitiligo. The observed *ACE* allele frequencies for I and D alleles were 0.56 and 0.44 in controls; and 0.61 and 0.39 in vitiligo patients, respectively (Table 1). The allele frequencies of *ACE* I/D polymorphism did not differ significantly between the controls and patient population ($P = 0.252$) (Table 1). The observed genotype frequencies of the vitiligo patients did not show significant difference as predicted by the Hardy–Weinberg equation ($P < 0.964$). Also the control population did not deviate from Hardy–Weinberg equilibrium ($P < 0.905$). This study has 86% statistical power for the effect size 0.2 to detect

Table 1. Distribution of alleles and genotypes for the I/D polymorphism in *ACE* intron 16 in Gujarat vitiligo patients and control population

		Observed genotype counts (%)			Observed allele frequencies		Expected genotype counts[a]			
	N	I/I	I/D	D/D	I	D	I/I	I/D	D/D	P-value
Controls	156	51 (32.7)	74 (47.4)	31 (19.9)	0.56	0.44	49.6	76.8	29.6	0.905
Patients	125	46 (36.8)	61 (48.8)	18 (14.4)	0.61	0.39	46.9	59.4	18.9	0.964
P-value			0.459[b]		0.252[c]					
Odds ratio (95% CI)	0.821 (0.585–1.151)									

[a]Observed versus expected according to the Hardy–Weinberg equation.
[b]Controls versus patients using the chi-square test with 3 × 2 contingency table.
[c]Controls versus patients using the chi-square test with 2 × 2 contingency table.

association of *ACE* I/D polymorphism at $P < 0.05$ in patients and control population.

Autoimmunity plays a major role in the pathogenesis of vitiligo (Kemp et al., 2001). Autoimmune diseases are also influenced by environmental factors in addition to genetic factors (Zhang et al., 2004). ACE and its related substrates or products are known to have various functions in the immune system (Vuk-Pavlovic and Rohrbach, 1988). However, its underlying pathogenic mechanism in autoimmune diseases is yet to be understood. Previously, the D allele of the *ACE* gene was reported to confer susceptibility to vitiligo in Korean population (Jin et al., 2004). However, the current study suggests that the polymorphic variant D allele is not associated with vitiligo susceptibility. This may be because of the differences in ethnicity and/or geographic location of the subjects under study. Our results are comparable to the previous report in which genotype frequencies for the I/D polymorphism were not significantly different between controls and generalized vitiligo patient population (Akhtar et al., 2005). Our results on the distribution rate of I/D > I/I > D/D in the control population are in accordance with the previously reported distribution rate of I/D for Chinese (Lee, 1994), Japanese (Ishigami et al., 1995) and Korean population (Jin et al., 2004). This is the first report on the association of the *ACE* polymorphism with vitiligo in Gujarat population where prevalence of vitiligo is alarmingly high, and interestingly, the *ACE* gene I/D polymorphism may not play a role in the development of generalized vitiligo in Gujarat population.

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Lack of genetic association of promoter and structural variants of mannan-binding lectin (*MBL2*) gene with susceptibility to generalized vitiligo

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Summary

Background Vitiligo is a common depigmenting disorder resulting from the loss of functional melanocytes in the skin. It is hypothesized to be of autoimmune origin. Mannan-binding lectin (MBL) plays an important role in innate immunity. It helps in the clearance of apoptotic cells and in complement activation. Genetic variability due to structural and promoter polymorphisms in the MBL2 gene has been reported to be associated with increased risk for several autoimmune diseases including vitiligo.

Objectives The aim of this study was to explore whether MBL2 structural and promoter polymorphisms are associated with generalized vitiligo in Gujarat where the prevalence of vitiligo is alarmingly high.

Materials and methods We undertook a case–control study to investigate the association of MBL2 gene exon 1 polymorphisms – codon 52, codon 54 and codon 57 as well as promoter −221 polymorphism in 92 patients with generalized vitiligo and 94 unaffected age-matched controls by polymerase chain reaction-heteroduplex analysis.

Results The genotype and allele frequencies of MBL2 structural and promoter polymorphisms did not differ significantly between the control and patient population (*P*-values: $P < 0{\cdot}019$ for codon 52, $P < 0{\cdot}373$ for codon 54, $P < 0{\cdot}855$ for codon 57 and $P < 0{\cdot}889$ for −221 promoter polymorphisms) after Bonferroni's correction for multiple testing, which suggests that there is no association of MBL2 structural and promoter polymorphisms with generalized vitiligo.

Conclusions Our results suggest that the well-documented structural and promoter polymorphisms of the MBL2 gene may not be associated with generalized vitiligo in the Gujarat population.

Vitiligo is an acquired hypomelanotic disorder characterized by circumscribed depigmented macules in the skin resulting from the loss of functional melanocytes from the cutaneous epidermis. This is a cosmetic disfigurement disorder and may lead to psychological and social problems, particularly in brown and black people. It affects 0·5–1% of the world population.[1] In India the incidence of vitiligo is found to be 0·5–2·5%[2] whereas Gujarat and Rajasthan states have the highest prevalence, ~8·8%.[3] The aetiology of vitiligo is still unknown, but it is hypothesized to be of autoimmune origin due to its frequent association with several autoimmune diseases, the presence of circulating autoantibodies and autoreactive T cells in the sera of patients with vitiligo compared with unaffected individuals.[4,5]

Various factors such as oxidative stress, genetic factors, neural factors, toxic metabolites and lack of melanocyte growth factors might contribute to precipitating the disease in susceptible people.[6] The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association.[7] It has been found that about 20% of patients with vitiligo have at least one first-degree relative affected.[8] Our study also showed that 22% of Gujarat vitiliginous patients exhibit positive family history and 14% of patients have at least one first-degree relative affected.[4] The disease does not follow the simple Mendelian inheritance pattern and its mode of heredity suggests that it is a polygenic disease.

Furthermore, several genes that play a role in regulating immunity have been associated with susceptibility to vitiligo

including allelic variants in the cytotoxic T-lymphocyte antigen-4 gene (*CTLA4*),[9–11] the autoimmune susceptibility loci *AIS1*, *AIS2*, *AIS3* and *SLEV1*,[12–14] the autoimmune regulator (*AIRE*) gene,[15] NACHT leucine-rich repeat protein 1 (*NALP1* – maps at *SLEV1* susceptibility locus),[16,17] protein tyrosine phosphatase nonreceptor type 22 (PTPN22)[18–20] and certain human leucocyte antigen specificities of the major histocompatibility complex.[21,22]

Mannan-binding lectin (MBL) [synonyms: mannose-binding lectin (MBL), mannose-binding protein (MBP), mannan-binding protein (MBP)] is a liver-derived calcium-dependent serum protein, which plays an important role in innate immune defence. MBL is a trimer of three identical polypeptides, and several trimers further combine to form a bouquet-like structure resembling C1q of the complement pathway. MBL binds to cell-surface ligands such as mannose, N-acetylglucosamine residues, which are expressed on a wide range of microorganisms. MBL binds to carbohydrate moieties on microorganisms and thus leads to activation of the complement system via the lectin pathway.[23] In addition to complement activation, the protein has several distinct functions including promotion of complement-independent opsonophagocytosis, modulation of inflammation and promotion of apoptosis.[24] The role of the MBL pathway in complement activation and in the clearance of apoptotic cells suggests that genetic variability in MBL may be involved in the pathogenesis of autoimmune diseases.[25] A possible role of MBL in vitiligo pathogenesis could be due to defective clearance of apoptotic cells, which may contribute substantially to the triggering of autoimmune responses.[26] Also, MBL is involved in inflammatory response, regulation of proinflammatory cytokines, controlling adhesion molecules on inflammatory cells and immune complex removal. Thus, these studies emphasize the importance of MBL in cutaneous autoimmunity.[27]

The functional *MBL2* gene is located on chromosome 10 (q11.2–q21) and comprises four exons. Exon 1 encodes the signal peptide, a cysteine-rich region and part of the glycine-rich collagenous region. Three functional single-nucleotide polymorphisms (SNPs) in exon 1 of the *MBL2* gene have been reported: codon 54 (GGC > GAC; designated B allele), codon 57 (GGA > GAA; designated C allele) and codon 52 (CGT > TGT; designated D allele).[28–30] These variant alleles in the collagen-like domain cause structural changes in MBL and will lead to its low plasma levels and thus results in plasma concentrations varying by more than 1000-fold within the population (5 ng mL^{-1} to 5 μg mL^{-1}).[31,32] The promoter region of the *MBL2* gene contains a number of regulatory elements, which affect transcription of the gene and hence polymorphisms in the promoter region, thus affecting the plasma MBL concentration directly. Besides these exon 1 variant alleles, SNPs at promoters −550 (G/C; allele L) and −221 (G/C; allele X) have also been associated with low plasma levels of MBL.[33]

Recently, an association between *MBL2* gene codon 54 (allele B) polymorphism and susceptibility to vitiligo has been described in a Turkish population.[34] The aim of this study was to investigate the association of MBL2-deficient genotypes of both promoter and exon 1 with vitiligo susceptibility in the Gujarat population.

Materials and methods

Study population

A total of 92 patients with generalized vitiligo (mean age 28·37 ± 1·740; 29 males and 63 females) and 94 unaffected age-matched controls (mean age 27·28 ± 0·841; 60 males and 34 females) of the Gujarat population participated in this study. Sixty-two patients and 72 age-matched controls were genotyped for codon 52 (allele D), 84 patients and 81 controls were genotyped for codon 54 (allele B), and 68 patients and 72 controls were genotyped for codon 57 (allele C). For genotyping of the −221 promoter polymorphism, 92 patients and 93 control subjects were used. The study plan was approved by the ethical committee of the Department of Biochemistry, M.S. University of Baroda, Vadodara, Gujarat. The importance of the study was explained to all participants and written consent was obtained from the patients.

Methods

Genomic DNA was prepared from venous blood of patients with vitiligo and controls using a 'whole blood DNA extraction kit' (Bangalore Genei, Bangalore, India) according to the manufacturer's instructions. The exon 1 structural and promoter polymorphisms were genotyped by the polymerase chain reaction (PCR)-heteroduplex analysis technique. This technique involves the use of a universal heteroduplex generator (UHG), a synthetic DNA molecule based on the target genomic sequence.[35] The genomic DNA PCR product was combined with the UHG-PCR product and the two were allowed to anneal to produce characteristic heteroduplexes for different alleles. These heteroduplexes displayed different electrophoretic mobilities on a polyacrylamide gel enabling identification of a subject's genotype.[35]

Polymerase chain reaction amplification of genomic DNA and universal heteroduplex generator

Two separate PCRs were performed on genomic DNA samples to amplify exon 1 and −221 (X/Y) promoter regions by using forward (5′-CCAACACGTACCTGGTTCC-3′) and reverse (5′-CTGTGACCTGTGAGGATG-C-3′) primers for exon 1 and forward (5′-AGGCATAAGCCAGCTGGCAAT-3′) and reverse (5′-CTAAGGAGGGGTTCATCTG-3′) primers for −221 (X/Y) promoter region.

All primers were synthesized from MWG-Biotech, Germany. The PCR was performed in a 20-μL system containing 50 ng genomic DNA, 0·25 mmol L^{-1} dNTPs, 20 pmol of each primer, 2 mmol L^{-1} $MgCl_2$, PCR gold buffer and 1·5 U Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.). PCR amplification was carried out in a thermal cycler

(ABI 9700; Applied Biosystems) with the following conditions: initial denaturation at 95 °C for 3 min followed by denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 7 min. For the synthesis of each UHG, the same PCR conditions were used with each 50 ng UHG template (template size of 133 bp for exon 1 and 111 bp for −221 promoter region), which are commercially synthesized UHG (MWG-Biotech AG, Ebersberg, Germany), specifically designed to detect the exon 1 and promoter regions of MBL2. PCR products were analysed on a 2% agarose gel prior to heteroduplexing and confirmed with 100 bp DNA ladder (MBI Fermentas, Burlington, ON, Canada). Confirmation of specific amplification of genomic and UHG DNA using 2% agarose gel electrophoresis enabled comparison of the relative amounts of DNA obtained from each sample reaction against those from the UHG reaction.

Heteroduplex analysis

A mixture of approximately equal amounts of genomic and UHG-PCR products were combined prior to heating to 95 °C for 5 min and then allowed to cool at room temperature over 2 h to enable greater specific annealing. The PCR mix was spun briefly and the appropriate volume of 6 × loading dye was added to each mix. Heteroduplexes thus formed were checked by 20% polyacrylamide gel electrophoresis followed by ethidium bromide staining (Figs 1, 2).

On heteroduplexing, homozygotes developed two heteroduplex bands and heterozygotes generated four heteroduplex bands (Figs 1, 2). The exception was WT/R52C, where only three bands were generated due to co-migration of two heteroduplexes as a single upper band[35] (WT designating one wild-type allele and R52C, G54D and G57E, indicating an allele with a mutation at codons 52, 54 and 57, respectively) (Fig. 1). Interpretation of band patterns was done manually with the aid of known samples used as a reference grid. The genotype WT/G57E generating four bands spanning the whole gel was taken as the reference, and was included in each run. The position of all the other bands could be judged using this sample as the reference.

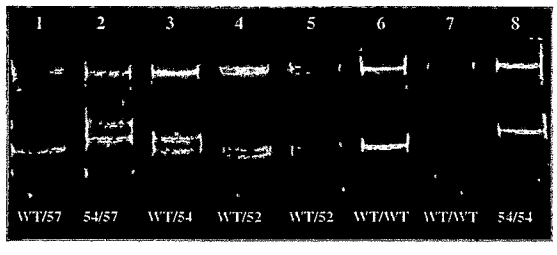

Fig 1. Polymerase chain reaction-heteroduplex analysis of MBL2 exon 1 (codon 52, codon 54 and codon 57) polymorphisms on 20% polyacrylamide gel electrophoresis: lane 1 shows heterozygous WT/57; lane 2 shows heterozygous 54/57; lane 3 shows heterozygous WT/54; lanes 4, 5 show heterozygous WT/52; lanes 6, 7 show homozygous WT/WT and lane 8 shows homozygous 54/54 individual.

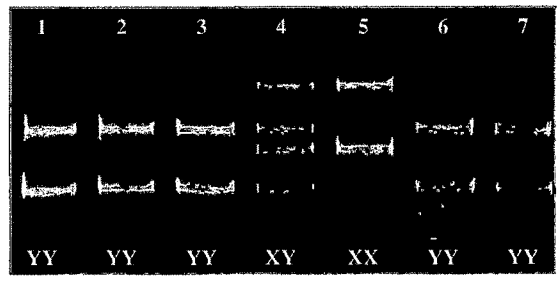

Fig 2. Polymerase chain reaction-heteroduplex analysis of MBL2 −221 (X/Y) promoter polymorphism on 20% polyacrylamide gel electrophoresis: lanes 1, 2, 3, 6 and 7 show homozygous YY; lane 4 shows heterozygous XY and lane 5 shows homozygous XX individual.

Statistical analysis

The distribution of the MBL2 genotypes and allele frequencies for patients and control subjects were compared using the χ^2 test with 3 × 2 and 2 × 2 contingency tables, respectively, using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, U.S.A.). The results were supported by odds ratios and 95% confidence intervals when appropriate. P-values < 0·0125 were considered as statistically significant due to Bonferroni's correction for multiple testing.

Results

We have investigated 92 patients with generalized vitiligo and 94 controls for exon 1 structural and promoter polymorphisms.

Genotyping of *MBL2* exon 1 structural polymorphisms

Codon 52, codon 54 and codon 57 polymorphisms of MBL2 exon 1 were not found to be associated in patients with generalized vitiligo ($P < 0·019$ for codon 52, $P < 0·373$ for codon 54, $P < 0·855$ for codon 57) when genotypes were compared with the χ^2 test 3 × 2 contingency table (Table 1). Also, there was no significant difference in allele frequencies of codon 52, codon 54 and codon 57 between patients and controls when compared with the 2 × 2 contingency table ($P < 0·020$ for D allele, $P < 0·378$ for B allele and $P < 0·858$ for C allele) (Table 1). Both the patient and control populations were found to be in Hardy–Weinberg equilibrium for all three exon 1 codon polymorphisms ($P < 1·000$ and $P < 0·932$, respectively, for codon 52 polymorphism; $P < 0·869$ and $P < 0·650$, respectively for codon 54 polymorphism; and $P < 0·932$ and $P < 0·909$, respectively, for codon 57 polymorphism) (Table 1). The genotypes for MBL2 structural polymorphisms were confirmed by running heteroduplexes on 20% polyacrylamide gel (Fig. 1).

The association study for exon 1 polymorphisms was also done by A/O genotyping where all three codon polymorphisms were placed in one group designated as 'O' allele and wild type as 'A' allele. The A/O genotypes were compared between controls and patients using the χ^2 3 × 2 contingency table.

SNP	Genotype or allele	Patients with vitiligo	Controls	P for association	P for HWE	Odds ratio (95% CI)
rs5030737, codon 52 (allele D)						
	Genotype	(n = 62)	(n = 72)			
	TT	0	0			
	CT	0	6	0·0192	1·0 (P); 0·932 (C)	0·08 (0·004–1·462)
	CC	62	65			
	Allele					
	T	0	8	0·007 (0·02[a])		
	C	124	136			
rs1800450, codon 54 (allele B)						
	Genotype	(n = 84)	(n = 81)			
	AA	1	0			
	AG	21	15	0·373	0·869 (P); 0·650 (C)	1·353 (0·694–2·639)
	GG	62	65			
	Allele					
	A	23	17	0·378		
	G	145	145			
rs1800451, codon 57 (allele C)						
	Genotype	(n = 68)	(n = 72)			
	AA	0	0			
	AG	6	7	0·855	0·932 (P); 0·909 (C)	0·899 (0·286–2·823)
	GG	62	65			
	Allele					
	A	6	7	0·858		
	G	130	137			
Genotype A/O[b]						
	Genotype	(n = 90)	(n = 94)			
	OO	1	1			
	AO	27	28	0·999	0·579 (P); 0·563 (C)	0·989 (0·566–1·726)
	AA	62	65			
	Allele					
	A	151	158	0·968		
	O	29	30			

[a]Yate's corrected P-value. SNP, single-nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium; [b]A, designation for wild-type alleles; O, common designation for the variant alleles B, C and D; (P), patients; (C), controls.

Table 1 Association studies for mannan-binding lectin gene (MBL2) exon 1 polymorphisms in Gujarat patients with generalized vitiligo

Interestingly, there was no significant association found between the A/O genotype and generalized vitiligo ($P < 0·999$) (Table 1). Furthermore, the 'O' allele was also not significantly associated with any of the population when compared using the 2×2 contingency table ($P < 0·968$) (Table 1).

Genotyping of *MBL2* –221 promoter polymorphism

In addition, we have also genotyped the –221 (X/Y) promoter polymorphism of the MBL2 gene in 92 patients and 93 controls. The genotypes for the –221 promoter polymorphism were confirmed by running heteroduplexes on 20% polyacrylamide gel (Fig. 2). There was no significant association found between the –221 promoter polymorphism and the risk of vitiligo when genotype frequencies of patients and controls were compared with the χ^2 test 3×2 contingency table ($P < 0·889$) (Table 2). Also, the allele frequencies of patients and controls for the –221 (X/Y) promoter polymorphism did not show significant difference when compared with the 2×2 contingency table ($P < 0·765$) (Table 2). However, both the control and patient populations were in accordance with Hardy–Weinberg equilibrium ($P < 0·120$ and $P < 0·370$, respectively) (Table 2).

Haplotype analysis

The promoter and structural genotypes of patients and controls were combined to construct haplotypes and to find out whether there was any haplotype associated with vitiligo susceptibility. Interestingly, no haplotype was found to be

Table 2 Distribution of alleles and genotypes for the −221 (X/Y; rs7096206) promoter polymorphism of MBL2 gene in patients with generalized vitiligo and controls

	n	Observed genotype counts (%)			Observed allele frequencies		Expected genotype counts[a]			
		X/X	X/Y	Y/Y	X	Y	X/X	X/Y	Y/Y	P-value
Controls	93	10 (10·75)	28 (30·10)	55 (59·13)	0·26	0·74	6·23	35·71	51·06	0·120
Patients	92	8 (8·70)	29 (31·52)	55 (59·78)	0·24	0·76	5·52	34·04	52·44	0·370
P-value			0·889[b]			0·765[c]				
OR (95% CI)			0·931 (0·582–1·489)							

[a]Observed vs. expected according to the Hardy–Weinberg equation; [b]controls vs. patients using the χ^2 test with 3 × 2 contingency table; [c]controls vs. patients using the χ^2 test with 2 × 2 contingency table. CI, confidence interval; OR, odds ratio.

Table 3 Distribution of mannan-binding lectin (MBL2) gene combined (structural + promoter) genotypes among patients with generalized vitiligo and controls

Combined genotype (structural + promoter)	Patients with vitiligo n = 90 (%)	Controls n = 88 (%)	P for association
YA/YA	33 (36·67)	34 (38·63)	
YA/XA	21 (23·34)	16 (18·18)	
YA/YO	20 (22·22)	19 (21·59)	
			0·962
XA/XA	8 (8·89)	9 (10·23)	
XA/YO	7 (7·78)	9 (10·23)	
YO/YO	1 (1·11)	1 (1·14)	
MBL high (YA/YA, YA/XA)	54 (60·00)	50 (56·82)	
MBL medium (XA/XA, YA/YO)	28 (31·11)	28 (31·82)	0·838
MBL low (XA/YO, YO/YO)	8 (8·89)	10 (11·36)	

MBL, mannan-binding lectin.

associated with generalized vitiligo when compared with the χ^2 test ($P < 0·962$) (Table 3).

Moreover, the haplotypes were distributed to represent MBL levels as high (YA/YA, YA/XA), medium (XA/XA, YA/YO) and low (XA/YO, YO/YO) in both controls and patients. However, no significant difference was found in the distribution of high, medium and low haplotypes when compared with the 2 × 2 contingency table ($P < 0·838$) (Table 3).

Discussion

Vitiligo susceptibility is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo as it shows frequent association with other autoimmune disorders such as rheumatoid arthritis, Graves' disease, etc.[36] One of the important regulators of innate immunity is MBL2, which is involved in complement activation and clearance of apoptotic cells suggestive of its importance in the pathogenesis of autoimmune diseases. We therefore selected MBL2 as a candidate gene to study vitiligo susceptibility in the Gujarat population and here we report that there is no association of MBL2 structural and promoter polymorphisms. Our previous studies on PTPN22 1858C/T (rs2476601) and angiotensin-converting enzyme (ACE) intron 16 I/D (NCBI: AF118569) polymorphisms did not show any significant association with vitiligo in the Gujarat population.[37,38]

MBL is a pattern-recognition molecule of the innate immune system and primarily recognizes specific sugar groups on the surface of microorganisms, enabling it to distinguish self from nonself. The three structural variants in exon 1 of the MBL2 gene are associated with significantly decreased levels of MBL compared with homozygotes of the wild-type gene.[39] The structural alterations in MBL due to three coding polymorphisms disrupt interactions of MBL with the associated serine proteases, thereby greatly diminishing complement-activating ability.[39,40]

The MBL concentration is also regulated by two additional promoter polymorphisms, namely −550 (G > C; allele L) and −221 (G > C; allele X), which directly affect the expression of the MBL gene. Because MBL is one of the proteins shown to be involved in the clearance of apoptotic cells, Werth *et al.*[26] suggested that any defect in the clearance of apoptotic cells may result in aggravation of autoimmune responses. In addition, MBL is also involved in inflammatory response, regulation of proinflammatory cytokines, controlling adhesion molecules on inflammatory cells and immune complex removal, thus emphasizing its importance in cutaneous autoimmunity.[27] Reports from Schallreuter *et al.*[41,42] showed a perturbed calcium homeostasis in vitiliginous melanocyte and keratinocyte cell cultures, suggesting that patients with vitiligo carrying functional and promoter polymorphisms may have defective calcium uptake resulting in low MBL concentration.

MBL2 gene polymorphisms have been associated with increased risk of autoimmune diseases such as systemic lupus erythematosus (SLE) in both Caucasoid and Chinese populations,[43,44] cutaneous lupus erythematosus and dermatomyositis.[26] Senaldi *et al.*[45] suggested that patients with SLE had a higher frequency of low levels of MBL than controls. Wang *et al.*[46] reported that codon 54 (allele B) of the MBL2 gene

might be associated with genetic susceptibility to Sjögren's syndrome (SJS). Thus most of the studies show that MBL deficiency is associated with poorer outcome as judged by clinical, inflammatory and radiographic indices.[47,48]

However, evaluation of MBL2 coding mutations in a Japanese population found no association with SLE and rheumatoid arthritis (RA).[49] Recent studies suggest that there is a greater prevalence of MBL deficiency in patients with RA[47] but other studies refute this assertion.[50] Rector *et al.*[51] found no association between structural polymorphisms in exon 1 of the MBL2 gene and susceptibility to Crohn disease.

Because vitiligo is hypothesized to be of autoimmune origin, it becomes relevant to screen patients with vitiligo for the presence of MBL2 structural and promoter variants which may predispose an individual for vitiligo. Previously, an association of codon 54 (allele B) with vitiligo was observed in a Turkish population where only two codons, 54 and 57, were genotyped with a smaller sample size.[34] Hence, it was pertinent to study all such important polymorphisms of MBL2 with an adequate sample size and we found there was no association between vitiligo and MBL2 structural and promoter polymorphisms. The positive association of codon 54 polymorphism and susceptibility to vitiligo described by Onay *et al.*[34] may be due to differences in the ethnicity of the studied populations.

This is the first report that shows nonassociation of structural and promoter polymorphisms of the MBL2 gene with generalized vitiligo. In conclusion, it can be considered that the structural and promoter polymorphisms in the MBL2 gene may not confer a role in generalized vitiligo susceptibility of the Gujarat population.

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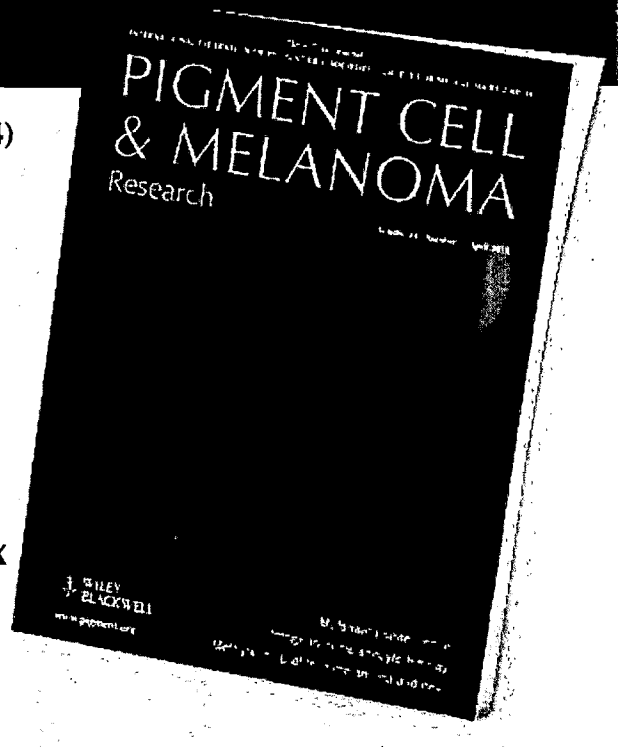

Cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) in isolated vitiligo: a genotype-phenotype correlation

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Cytotoxic T-lymphocyte-associated antigen-4 (*CTLA*-4) in isolated vitiligo: a genotype-phenotype correlation

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Dear Editor,

CTLA-4 is a negative regulator of T-cell function, which is suggested to be involved in susceptibility to autoimmune diseases including vitiligo, an acquired pigmentary disorder resulting from loss of melanocytes. Genetic variability in *CTLA*-4 gene is associated with its altered levels. *CTLA*-4 is expressed on activated $CD4^+$ and $CD8^+$ T cells and binds the same ligands, B7-1 and B7-2, as CD28 but with a 20–50-fold higher affinity. The CTLA-4:B7 interaction plays a critical role in regulating self-tolerance, and hence susceptibility to autoimmune diseases. The human *CTLA*-4 gene encodes two transcripts of 1.8 and 0.8 kb, known as full length *CTLA*-4 (fl*CTLA*-4) and soluble *CTLA*-4 (s*CTLA*-4) (Harper et al., 1991).

The aim of study was to explore exon 1 +49A/G (rs231775) and 3′ UTR CT60A/G (rs3087243) single nucleotide polymorphisms (SNPs) in *CTLA*-4 gene and to correlate them with *CTLA*-4 transcript levels in vitiligo patients and controls in Gujarat, India. The PCR-RFLP technique was used to genotype these polymorphisms (Supporting Information Figure S1) in 437 vitiligo patients and 746 controls. s*CTLA*-4 and fl*CTLA*-4 mRNA expression was assessed in 76 patients and 83 controls by real time PCR (Supporting Information Data S1, Tables S1 and S2).

CTLA-4 +49A/G polymorphism was not associated with vitiligo patients (P = 0.771); however, CT60A/G polymorphism showed a significant association (P = 0.0002) when genotypes were compared with a Chi-squared test 3 × 2 contingency table (Table 1). There was also no significant difference in the allele frequencies of +49A/G polymorphism (P = 0.461), whereas a significant difference in allele frequencies of CT60A/G polymorphism was observed (P = 0.0002) between patients and controls when compared with a 2 × 2 contingency table (Table 1). Both patient and control populations were in Hardy–Weinberg equilibrium for +49A/G polymorphism (P = 0.272 and P = 0.126, respectively). The control population was in Hardy–Weinberg equilibrium for CT60A/G polymorphism, whereas the patient population deviated from the equilibrium (P = 0.075 and P < 0.0001) (Table 1). This study has 95% statistical power for the effect size 0.1 to detect association of both the polymorphisms of *CTLA*-4 at P < 0.05 (Faul et al., 2007).

The two polymorphisms investigated were in moderate linkage disequilibrium (LD) (+49A/G: CT60A/G; $D' = 0.64$, $r^2 = 0.11$) as determined by HAPLOVIEW program version 4.1 (Barrett et al., 2005). The estimated frequencies of the haplotypes did not differ between patients and controls (global P-value = 0.123). However, the GG haplotype was more frequently observed in patients and increased the risk of vitiligo 1.2-fold [P = 0.048; odds ratio (OR): 1.243; 95% confidence interval (95%CI): (1.001–1.543)] (Supporting Information Table S3).

Expression of fl*CTLA*-4 and s*CTLA*-4 mRNA showed a significant decrease in patients after normalization with *GAPDH* expression (P = 0.007 and P = 0.037, respectively) (Figure 1A). The $2^{-\Delta\Delta Cp}$ analysis showed an approximately two-fold change in the expression of fl*CTLA*-4 and s*CTLA*-4 mRNA expression in patients as compared with controls (Figure 1B). The expression levels of fl*CTLA*-4 and s*CTLA*-4 for AA, AG and GG genotypes of +49A/G polymorphism did not differ significantly (Supporting Information Figure S2A,B). The expression levels of both fl*CTLA*-4 and s*CTLA*-4 differed significantly between patients and controls for CT60GG genotype (P = 0.004 and P = 0.005, respectively) but not for CT60AA genotype (P = 0.343 and P = 0.205, respectively) (Figure 1C,D).

The expression levels of fl*CTLA*-4 and s*CTLA*-4 were also analyzed with respect to haplotypes. Both fl*CTLA*-4 and s*CTLA*-4 mRNA levels were significantly decreased with AG haplotypes in patients as compared to controls (P = 0.036 and P = 0.020, respectively). The other three haplotypes, AA, GA and GG, did not differ with respect to fl*CTLA*-4 and s*CTLA*-4 expression levels (Figure 2A,B). We could not detect any significant difference in the s*CTLA*-4/fl*CTLA*-4 mRNA expression ratio between patients and controls (P = 0.346) (Figure S2C). However, +49AG genotype showed a decreased mRNA expression

Table 1. Association studies for *CTLA-4* gene exon 1 +49A/G and 3′ UTR CT60A/G polymorphisms in Gujarat vitiligo patients

SNP	Genotype or allele	Vitiligo patients (Freq.)	Controls (Freq.)	P for association	P for HWE	Odds ratio (95%CI)
	Genotype	(n = 347)	(n = 746)			
rs231775	AA	169 (0.49)	347 (0.47)	0.771	0.272 (Ps)	0.930 (0.766–1.128)
Exon 1	AG	140 (0.40)	310 (0.41)		0.126 (C)	
(+49A/G)	GG	38 (0.11)	89 (0.12)			
	Allele					
	A	478 (0.69)	1004 (0.67)	0.461		
	G	216 (0.31)	488 (0.33)			
	Genotype	(n = 437)	(n = 738)			
rs3087243	AA	146 (0.33)	256 (0.35)	0.0002*	<0.0001 (Ps)	1.417 (1.198–1.677)
3′ UTR	AG	135 (0.31)	337 (0.46)		0.075 (C)	
(CT60A/G)	GG	156 (0.36)	145 (0.19)			
	Allele					
	A	427 (0.49)	849 (0.58)	0.0002*		
	G	447 (0.51)	627 (0.42)			

n, number of patients/controls; HWE, Hardy–Weinberg equilibrium; Ps, patients; and C, controls. Values are significant at $P \leq 0.05$.
*Bonferroni's corrected P-value.

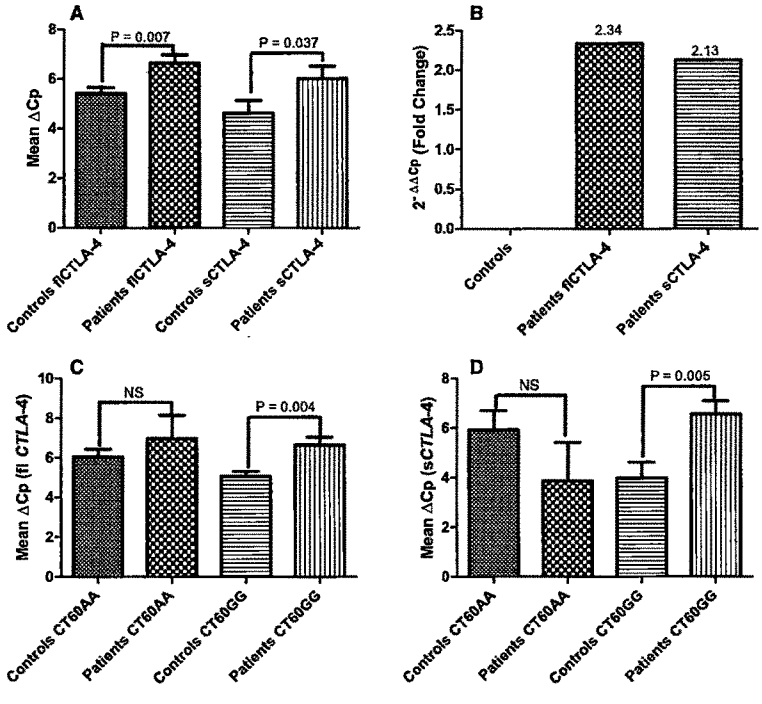

Figure 1. Relative gene expression of fl*CTLA-4* and s*CTLA-4* in 83 controls and 76 vitiligo patients. (A) Expression of fl*CTLA-4* and s*CTLA-4* mRNA as determined by mean ΔCp. Vitiligo patients showed significantly reduced mRNA levels of fl*CTLA-4* (P = 0.007) and s*CTLA-4* (P = 0.037) as compared to controls. (B) Expression fold change of fl*CTLA-4* and s*CTLA-4* showed approximately two-fold change as determined by the $2^{-\Delta\Delta Cp}$ method. (C) Relative mRNA expression of fl*CTLA-4* with respect to CT60A/G genotype. GG genotype showed a significant decrease in the levels of fl*CTLA-4* mRNA (P = 0.004) in patients as compared with AA genotype (P = 0.343) as determined by mean ΔCp. (D) Relative mRNA expression of s*CTLA-4* with respect to CT60A/G genotypes. GG genotype showed a significant decrease in levels of s*CTLA-4* mRNA (P = 0.005) in patients as compared with AA genotype (P = 0.205) as determined by mean ΔCp. NS, non-significant.

ratio in patients (P = 0.049), whereas the other two genotypes, +49AA and +49GG, did not differ (Figure 2C). When mRNA expression ratios for CT60A/G genotypes were compared, GG genotype showed a significant decrease in the ratio (P = 0.019) in patients; however, AA genotype did not differ significantly (Figure 2D). Moreover, the ratio of s*CTLA-4* and fl*CTLA-4* mRNA expression did not differ significantly for any of the haplotypes (AA, AG, GA, GG) (Figure S2D).

A few studies have addressed the possible functional significance of *CTLA-4* polymorphisms. Interestingly, the G allele of *CTLA-4* +49A/G SNP was reported to be involved in the altered intracellular transport of CTLA-4 protein and its availability on the cell surface. In addition, the 3′ UTR CT60A/G allelic variation was reported to be correlated with lower mRNA levels of the soluble alternative splice form of *CTLA-4* (s*CTLA-4*) (Ueda et al., 2003), which is in concordance with our results.

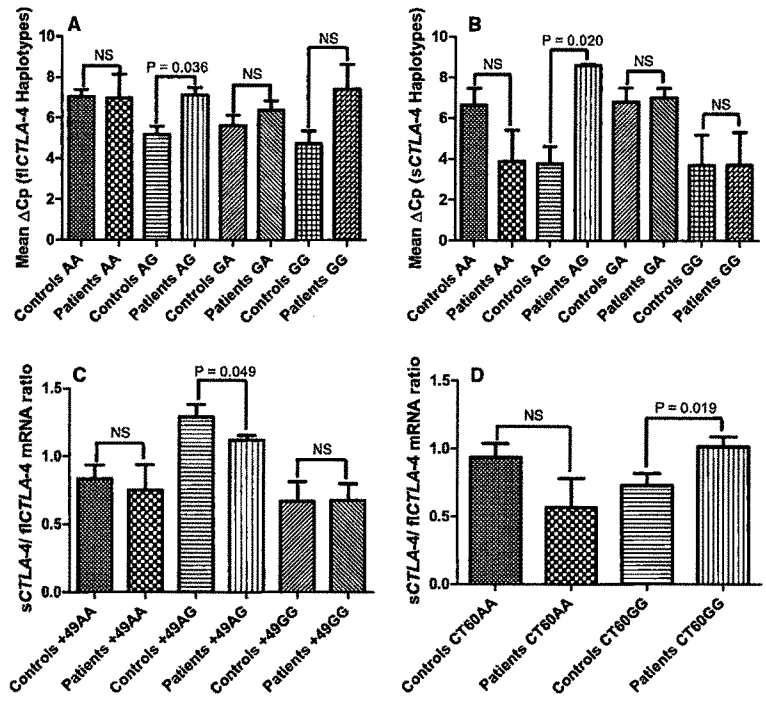

Figure 2. Relative mRNA expression of fl*CTLA*-4 and s*CTLA*-4 with respect to haplotypes and s*CTLA*-4/fl*CTLA*-4 mRNA ratios in 83 controls and 76 vitiligo patients. (A) AG haplotype showed a significant decrease in the levels of fl*CTLA*-4 mRNA in patients as compared with controls as determined by mean ΔCp (P = 0.036). The other three haplotypes, AA, GA and GG, did not differ in fl*CTLA*-4 expression in patients and controls (P = 0.955, P = 0.476 and P = 0.075, respectively). (B) AG haplotype showed a significant decrease in the levels of s*CTLA*-4 mRNA in patients as compared with controls as determined by mean ΔCp (P = 0.020). The other three haplotypes, AA, GA and GG, did not differ in fl*CTLA*-4 expression in patients and controls (P = 0.152, P = 0.865 and P = 0.992, respectively). (C) The s*CTLA*-4/fl*CTLA*-4 mRNA ratio was analyzed with respect to +49A/G genotypes in patients and controls. AG genotype showed a significant decrease in the ratio (P = 0.049) as compared to AA (P = 0.668) and GG (P = 0.964) genotypes. (D) The s*CTLA*-4/fl*CTLA*-4 mRNA ratio was analyzed with respect to CT60A/G genotypes in patients and controls. GG genotype showed a significant increase in the ratio (P = 0.019) as compared with AA (P = 0.096) genotype. NS, non-significant.

In the present study, we report an association between CT60A/G polymorphism and susceptibility to vitiligo. However, studies from Caucasian and Romanian populations did not find this association with vitiligo (Birlea et al., 2009; LaBerge et al., 2008). Another study from a Caucasian population only showed a significant association of the polymorphism with vitiligo patients who had other autoimmune diseases (Blomhoff et al., 2005). We did not find an association of genotype and allele frequencies for +49A/G polymorphism with vitiligo patients and our results are in line with those of Romanian and South Indian populations (Birlea et al., 2009; Deeba et al., 2010).

This is the first report where *CTLA*-4 expression levels were determined in vitiligo patients. Interestingly, we found decreased mRNA expression of both fl*CTLA*-4 and s*CTLA*-4 in patients and CT60G allele greatly reduced the mRNA expression of both isoforms, suggesting its crucial role in the pathogenesis of vitiligo. CT60AA genotype did not modulate *CTLA*-4 mRNA expression, suggesting that there may be other genetic factors involved in disease pathogenesis in patients harboring this genotype, evidence that vitiligo may have varied types of precipitating factors.

Further, decreased s*CTLA*-4 mRNA levels in patients with haplotype AG (+49A:CT60G) indicates the positive correlation of CT60G in vitiligo pathogenesis. Recently, Gerold et al. (2011) showed decreased s*CTLA*-4 levels in Type 1 diabetes, suggesting that lower s*CTLA*-4 expression may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. Moreover, an increased s*CTLA*-4/fl*CTLA*-4 mRNA ratio with +49AG and CT60GG genotypes in vitiligo patients suggests that G allele confers lower s*CTLA*-4 mRNA levels. These results are in agreement with those of Atabani et al. (2005). In contrast, Anjos et al. (2005) and Mayans et al. (2007) could not find an effect of the CT60 polymorphism on the expression of s*CTLA*-4 and fl*CTLA*-4.

In conclusion, our findings suggest that the dysregulated *CTLA*-4 expression in vitiligo patients could result, at least in part, from variations at the genetic level. For the first time, we show that the 3′ UTR CT60A/G poly-

morphism of the *CTLA-4* gene influences both full length and soluble *CTLA-4* mRNA levels in vitiligo patients. This genotype–phenotype correlation of *CTLA-4* supports the autoimmune pathogenesis of vitiligo.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. PCR-RFLP analysis of *CTLA-4* exon 1 +49 A/G and 3′ UTR CT60A/G polymorphisms.

Figure S2. Relative mRNA expression of fl*CTLA-4*, s*CTLA-4* and its ratios (s*CTLA-4*/fl*CTLA-4*) in 83 controls and 76 vitiligo patients.

Table S1. Demographic characteristics of vitiligo patients and unaffected controls.

Table S2. Primers used for *CTLA-4* SNPs genotyping and gene expression analysis.

Table S3. Distribution of haplotypes frequencies for *CTLA-4* gene polymorphisms (+49A/G and CT60A/G) among vitiligo patients and controls.

Data S1. Methods.

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ORIGINAL ARTICLE

See related commentary on pg 13

HLA Alleles and Amino-Acid Signatures of the Peptide-Binding Pockets of HLA Molecules in Vitiligo

Archana Singh[1], Pankaj Sharma[2], Hemanta K. Kar[2], Vinod K. Sharma[3], Manoj K. Tembhre[3], Somesh Gupta[3], Naresh C. Laddha[4], Mitesh Dwivedi[4], Rasheedunnisa Begum[4], The Indian Genome Variation Consortium[5,6], Rajesh S. Gokhale[1,5] and Rajni Rani[1]

Vitiligo is a depigmenting disorder of the skin that is characterized by the loss of functional melanocytes from the lesional sites. Although the exact etiology is not understood, autoimmunity is thought to be a crucial deterministic factor. A recurring theme of several autoimmune disorders is the aberrant presentation of self-antigens to the immune system, which triggers downstream perturbations. Here we examine the role of alleles of HLA class I and class II loci to delineate vitiligo manifestation in two distinct populations. Our studies have identified three specific alleles, *HLA-A*33:01*, *HLA-B*44:03*, and *HLA-DRB1*07:01*, to be significantly increased in vitiligo patients as compared with controls in both the initial study on North Indians ($N=1,404$) and the replication study in Gujarat ($N=355$) cases, establishing their positive association with vitiligo. Both generalized and localized vitiligo have the same predisposing major histocompatibility complex alleles, i.e., *B*44:03* and *DRB1*07:01*, in both the populations studied, beside the differences in the frequencies of other alleles, suggesting that localized vitiligo too may be an autoimmune disorder. Significant differences in the amino-acid signatures of the peptide-binding pockets of HLA-A and HLA-B α-chain and HLA-DR β-chain were observed between vitiligo patients and unaffected controls.

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INTRODUCTION

Vitiligo is a multifactorial, depigmenting disorder of the skin characterized by loss of functional melanocytes, resulting in appearance of milky white patches on the skin. It is the most common pigmentation disorder affecting 0.5–1% of the world population (Taieb and Picardo, 2007). In India, the incidence varies from 0.25 to 2.5% (Das *et al.*, 1985; Handa and Kaur, 1999) in most ethnic groups, with 8.8% in populations from western states of Gujarat and Rajasthan (Shajil *et al.*, 2006). Vitiligo manifests in broadly two clinical subtypes: generalized and localized (Taieb and Picardo, 2007). Generalized vitiligo includes vitiligo vulgaris, acrofacial vitiligo, and vitiligo universalis. Localized vitiligo includes segmental and focal types (Taieb and Picardo, 2007). Precise etiology of vitiligo is not known; however, several hypotheses have been proposed, which include autoimmune (Kemp *et al.*, 2001), neural (Dell'anna and Picardo, 2006), autocytotoxic (Lerner, 1971), and genetic hypotheses (Bhatia *et al.*, 1992). Of these, autoimmune hypothesis remains most widely accepted because of frequent occurrence of other autoimmune diseases in vitiligo cases, the presence of autoreactive T cells in the vitiligenous lesions and peripheral circulation, and the presence of circulating autoantibodies in the sera of the patients (Schwartz and Janniger, 1997; Kovacs, 1998; Kemp *et al.*, 2001; Lang *et al.*, 2001; Wankowicz-Kalinska *et al.*, 2003; van den Boorn *et al.*, 2009; Waterman *et al.*, 2010). The frequency of vitiligo cases in first-degree relatives is ~7%, suggesting an important role of genetic factors in disease pathogenesis (Majumder *et al.*, 1993; Alkhateeb *et al.*, 2003).

The major histocompatibility complex (MHC) has been implicated in recent genome-wide association studies on vitiligo (Jin *et al.*, 2010; Quan *et al.*, 2010), in which several single-nucleotide polymorphisms in the MHC region were significantly associated with the disease. However, the authors imputed the HLA (MHC of man) *class I* alleles, based on the linkage disequilibrium (LD) of *HLA* alleles with the specific single-nucleotide polymorphisms, but could not impute *HLA class II* alleles because of limitations of *HLA* allele imputation in the Chinese Han from Beijing (China) samples (Quan *et al.*, 2010). MHC spans about 3.6 megabases on chromosome 6p21.3 of man (Horton *et al.*, 2004), with 224 known gene loci. *HLA* is the most

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Abbreviations: CI, confidence interval; MHC, major histocompatibility complex; OR, odds ratio

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polymorphic system of the human genome, with 1,519 alleles for *HLA-A* locus, 2,069 alleles of *HLA-B* locus, 1,016 alleles for HLA-C locus, and 966 alleles for *HLA-DRB1*. With such a great diversity at each locus, LD of *HLA* alleles with diallelic single-nucleotide polymorphisms may be misleading, as several *HLA* alleles may be in LD with one or the alternate single-nucleotide polymorphism. Thus, the derivation based on these LDs may not be accurate, especially in the populations from which no previous *HLA* data are available. Thus, it is important to study the frequencies of HLA alleles in patients rather than imputing based on LD.

Many autoimmune diseases such as Type 1 diabetes (Todd *et al.*, 1987; Rani *et al.*, 2004) and rheumatoid arthritis (Winchester, 1994) have been shown to be associated with specific alleles of one or more genes of the *MHC*. The association of multiple *HLA* class I and class II antigens have been suggested for vitiligo (de Vijlder *et al.*, 2004; Zhang *et al.*, 2004a, b), although no consensus could be reached, because of distinct ethnic groups (Zamani *et al.*, 2001; Tastan *et al.*, 2004; Orozco-Topete *et al.*, 2005), small sample sizes, and low-resolution typing methods used to identify the HLA antigens. We report here a very robust study on a sample size of 1,404 vitiligo cases and 902 unaffected controls from North India for the alleles at *HLA-A*, *-B*, and *-C* (class I), and *-DRB1* (class II) loci using molecular methods. The replication study was conducted on 355 cases and 441 controls from Gujarat (Table 1). We also report differences in the amino-acid signatures of peptide-binding pockets of the HLA molecules in vitiligo patients as compared with controls.

RESULTS

HLA class I and *II* alleles and haplotypes in vitiligo cases as compared with unaffected controls

Diversity in the number and frequencies of HLA alleles was observed both in patients and controls. We observed 78, 160, 35, and 106 alleles for *HLA-A*, *-B*, *-C*, and *-DRB1* loci, respectively, in cases, and 68, 111, 44, and 94 alleles for *HLA-A*, *-B*, *-C*, and *-DRB1* loci, respectively, in unaffected controls from North India, and similar diversity was observed in the replication study. However, *HLA-A*33:01*, *B*44:03*, *C*07:01*, *DRB1*07:01*, and *DRB1*12:02* were significantly increased, and *HLA-A*26:01*, *A*31:01*, *B*08:01*, *B*51:01*, *C*12:03*, *DRB1*03:01*, *DRB1*11:01*, and *DRB1*15:02* were significantly reduced (Table 2 and Supplementary Table S1 online) in the vitiligo subjects from North India as compared with controls. In the replication study, we observed *HLA-A*33:01*, *B*44:03*, and *DRB1*07:01* to be significantly increased, and *DRB1*03:01* and *DRB1*11:01* to be significantly reduced in the cases as compared with ethnically matched controls (Table 2). These associations were significant, irrespective of the age at onset or the gender of the cases (Supplementary Tables S2–S5 online).

To determine which *HLA* alleles were significantly associated with vitiligo in the absence of *DRB1*07:01*, we deleted all the samples with *DRB1*07:01* from both the case and control groups and then analyzed the data. *B*37:01* and *DRB1*10:01* were significantly increased, whereas *B*08:01* and *DRB1*03:01* (Table 3) were significantly reduced in vitiligo cases in both initial and replication studies. In *A*33:01*-negative cases, *B*37:01*, *B*44:03*, *DRB1*07:01*, and *DRB1*10:01* were significantly increased and *DRB1*03:01* was significantly reduced as compared with *A*33:01*-negative controls in both initial and replication studies (Supplementary Table S6 online). In *B*44:03*-negative cases too, we observed a significant increase of *B*37:01*, *DRB1*07:01*, and *DRB1*10:01* and a significant decrease of *DRB1*03:01* as compared with *B*44:03*-negative controls in both the populations studied (Supplementary Table S7 online). *DRB1*07:01* remained significantly increased in *B*37:01*-negative patient samples also from North India ($P<5.69\times10^{-33}$, odds ratio (OR) = 3.03, 95% confidence interval (CI) = 2.51–3.66) and Gujarat ($P<1.33\times10^{-17}$, OR = 3.87, 95% CI = 2.78–5.39, data not shown).

HLA-C alleles were studied only in North Indian vitiligo cases ($N=404$) and healthy controls ($N=438$). We did not see any significant increase in *HLA-C* group 1 and group 2 alleles (based on Ser^{77}/Asn^{80} and Asn^{77}/Lys^{80}, respectively) in vitiligo as compared with controls (Supplementary Table S1 online). In the absence of *C*07:01*, which was significantly increased in vitiligo cases, *DRB1*07:01* still remained significantly increased (30% in vitiligo vs. 23.08% in controls) in cases as compared with controls ($P<0.047$, OR = 1.42, 95% CI = 0.99–2.06). However, in *DRB1*07:01*-negative samples, *C*07:01* does not remain significant (14.09% in vitiligo vs. 8.81% in controls) in vitiligo cases when compared with controls ($P<0.053$, OR = 1.69, 95% CI = 0.95–3.03), suggesting that association of *C*07:01* is probably because of it being in LD with *DRB1*07:01*.

Estimation of *HLA* haplotype frequencies was done using the expectation maximization algorithm (Excoffier and Slatkin, 1995; Excoffier *et al.*, 2005) with Arlequin Ver 3.5

Table 1. Description of vitiligo patients and controls used in the study

	Unaffected controls		Vitiligo patients	
Population	Unaffected controls (males, females, age mean ± SD)	Total vitiligo patients (males, females, age mean ± SD, age at onset mean ± SD)	Generalized vitiligo (males, females, age mean ± SD, age at onset mean ± SD)	Localized vitiligo (males, females, age mean ± SD, age at onset mean ± SD)
North India	902 (538, 364, 36.35 ± 14.75)	1,404 (838, 566, 28.72 ± 14.59, 22.15 ± 14.32)	1,097 (649, 448, 29.44 ± 15.04, 22.34 ± 14.61)	307 (189, 118, 26.16 ± 12.50, 21.46 ± 13.22)
Gujarat (replication study)	441 (264, 177, 28.04 ± 14.97)	355 (148, 207, 31.18 ± 15.03, 15.70 ± 12.65)	250 (107, 143, 32.06 ± 14.82, 16.14 ± 12.61)	105 (41, 64, 29.12 ± 15.37, 14.66 ± 12.76)

Table 2. *HLA-A*, *HLA-B*, and *HLA-DRB1* alleles showing significant differences in vitiligo patients as compared with unaffected controls from North India and Gujarat

	Vitiligo	Generalized	Localized	Controls	Vitiligo versus unaffected controls		Generalized vitiligo versus controls		Localized vitiligo versus controls	
HLA allele	No. (%)	No. (%)	No. (%)	No. (%)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
A*02:01 N India	197 (14.03)	160 (14.59)	37 (12.05)	88 (9.76)	0.002	1.50 (1.14–1.98)	0.001	1.57 (1.18–2.10)	0.25	1.26 (0.82–1.93)
Gujarat	27 (7.62)	17 (6.80)	10 (9.62)	44 (10.78)	0.13	0.68 (0.40–1.15)	0.087	0.60 (0.32–1.11)	0.72	0.88 (0.38–1.86)
*A*26:01* N India	83 (5.91)	64 (5.83)	19 (6.19)	90 (9.98)	0.00029	0.56 (0.41–0.78)	0.0005	0.56 (0.39–0.79)	0.045	0.59 (0.34–1.00)
Gujarat	21 (5.93)	15 (6.0)	6 (5.76)	36 (8.82)	0.13	0.65 (0.35–1.17)	0.18	0.66 (0.33–1.27)	0.31	0.63 (0.21–1.57)
*A*31:01* N India	43 (3.06)	32 (2.92)	11 (3.58)	58 (6.43)	0.0001	0.45 (0.30–0.70)	0.0002	0.43 (0.27–0.69)	0.063	0.54 (0.25–1.05)
Gujarat	16 (4.51)	13 (5.2)	3 (2.88)	18 (4.41)	0.94	1.02 (0.48–2.16)	0.64	1.18 (0.53–2.61)	0.35[1]	0.72 (0.31–1.65)[2]
*A*33:01* N India	353 (25.14)	276 (25.16)	77 (25.08)	104 (11.53)	1.21×10^{-15}	2.57 (2.02–3.29)	1.09×10^{-14}	2.57 (2.00–3.33)	9.01×10^{-9}	2.56 (1.82–3.61)
Gujarat	69 (19.49)	53 (21.2)	16 (15.38)	51 (12.5)	0.008	1.69 (1.12–2.56)	0.003	1.88 (1.20–2.93)	0.43	1.27 (0.65–2.40)
*A*68:01* N India	189 (13.46)	161 (14.68)	28 (9.12)	139 (15.41)	0.19	0.85 (0.66–1.09)	0.64	0.94 (0.73–1.21)	0.0058	0.55 (0.34–0.85)
Gujarat	71 (20.05)	55 (22.0)	16 (15.38)	46 (11.27)	0.0008	1.97 (1.29–3.02)	0.0002	2.21 (1.41–3.49)	0.25	1.43 (0.72–2.71)
*B*08:01* N India	68 (4.84)	53 (4.83)	15 (4.89)	112 (12.42)	3.68×10^{-11}	0.35 (0.25–0.49)	8.63×10^{-10}	0.35 (0.25–0.50)	0.0002	0.36 (0.19–0.64)
Gujarat	27 (7.64)	17 (6.85)	10 (9.52)	38 (9.33)	0.40	0.80 (0.46–1.38)	0.26	0.71 (0.37–1.33)	0.95	1.02 (0.43–2.18)
*B*37:01* N India	95 (6.77)	76 (6.93)	19 (6.19)	41 (4.55)	0.027	1.54 (1.03–2.27)	0.023	1.56 (1.04–2.37)	0.25	1.38 (0.76–2.49)
Gujarat	43 (12.18)	29 (11.69)	14 (13.33)	26 (6.38)	0.0056	2.03 (1.18–3.52)	0.018	1.94 (1.07–3.52)	0.018	2.25 (1.04–4.68)
*B*44:03* N India	419 (29.84)	347 (31.63)	72 (23.45)	97 (10.75)	7.05×10^{-27}	3.53 (2.76–4.53)	5.41×10^{-29}	3.83 (2.98–4.96)	2.98×10^{-8}	2.54 (1.78–3.61)
Gujarat	88 (24.92)	66 (26.61)	22 (20.95)	39 (9.58)	1.54×10^{-8}	3.13 (2.04–4.84)	8.29×10^{-9}	3.42 (2.17–5.42)	0.001	2.50 (1.33–4.58)
*B*51:01* N India	133 (9.47)	114 (10.39)	19 (6.19)	167 (18.51)	3.00×10^{-10}	0.46 (0.35–0.59)	2.00×10^{-7}	0.51 (0.39–0.66)	2.34×10^{-7}	0.29 (0.17–0.48)
Gujarat	46 (13.03)	33 (13.30)	13 (12.38)	45 (11.05)	0.40	1.20 (0.76–1.91)	0.38	1.23 (0.74–2.04)	0.70	1.13 (0.54–2.25)
*DRB1*03:01* N India	92 (6.55)	67 (6.11)	25 (8.14)	174 (19.29)	9.23×10^{-21}	0.29 (0.22–0.38)	2.10×10^{-19}	0.27 (0.19–0.37)	5.37×10^{-6}	0.37 (0.23–0.58)
Gujarat	22 (6.19)	14 (5.6)	8 (7.61)	61 (13.83)	0.00045	0.41 (0.23–0.70)	0.0008	0.37 (0.18–0.68)	0.08	0.51 (0.20–1.12)
*DRB1*07:01* N India	724 (51.57)	601 (54.79)	123 (40.07)	248 (27.49)	3.16×10^{-30}	2.80 (2.33–3.37)	1.10×10^{-34}	3.19 (2.63–3.87)	0.000037	1.76 (1.33–2.33)
Gujarat	184 (51.83)	133 (53.2)	51 (48.57)	111 (25.17)	9.81×10^{-15}	3.19 (2.34–4.36)	1.28×10^{-13}	3.37 (2.39–4.75)	2.38×10^{-6}	2.80 (1.76–4.45)
*DRB1*11:01* N India	87 (6.20)	64 (5.83)	23 (7.49)	93 (10.31)	0.0003	0.57 (0.41–0.78)	0.0002	0.53 (0.38–0.76)	0.14	0.70 (0.42–1.15)
Gujarat	19 (5.35)	16 (6.4)	3 (2.85)	57 (12.92)	0.0003	0.38 (0.21–0.67)	0.0073	0.46 (0.24–0.84)	0.0010[1]	0.22 (0.10–0.49)[2]
*DRB1*12:02* N India	67 (4.77)	53 (4.83)	14 (4.56)	14 (1.55)	0.00004	3.17 (1.75–6.16)	0.00005	3.22 (1.74–6.32)	0.0025	3.03 (1.32–6.93)
Gujarat	14 (3.94)	11 (4.4)	4 (3.80)	23 (5.21)	0.51	0.80 (0.38–1.63)	0.63	0.83 (0.36–1.82)	0.38[1]	0.78 (0.38–1.63)[2]
*DRB1*15:02* N India	165 (11.75)	117 (10.67)	48 (15.64)	152 (16.85)	0.0005	0.65 (0.51–0.84)	0.00005	0.59 (0.45–0.77)	0.62	0.91 (0.63–1.31)
Gujarat	54 (15.21)	37 (14.8)	17 (16.19)	82 (18.59)	0.20	0.78 (0.52–1.16)	0.20	0.76 (0.48–1.18)	0.56	0.84 (0.44–1.52)

Abbreviations: CI, confidence interval; N, North; OR, odds ratio.
[1]*P*-value calculated using Fisher's exact test.
[2]OR was calculated by using Woolf's method with Haldane's modification.
Vitiligo patient samples from North India (*N*=1,404; generalized (*n*=1,097), localized (*n*=307)) were compared with unaffected controls (*N*=902, North Indian origin) for *HLA-A*, *HLA-B*, and *HLA-DRB1* loci. Overall, 354 vitiligo patients, 250 generalized and 104 localized, were compared with 408 Gujarati controls for *HLA-A* locus. Overall, 353 vitiligo patients, 248 generalized and 105 localized, were compared with 407 Gujarati controls for *HLA-B* locus, and 355 vitiligo patients, 250 generalized and 105 localized, were compared with 441 Gujarati controls for *HLA-DRB1* locus.

(http://cmpg.unibe.ch/software/arlequin35/). Haplotype analysis for three loci showed that haplotypes *A*33:01-B*44:03-DRB1*07:01* and *A*24:02-B*44:03-DRB1*07:01* were significantly increased, and *A*26:01-B*080:01-DRB1*03:01* was significantly reduced in vitiligo patients in both initial and the replication studies (Table 4). Analysis of two-locus haplotypes revealed that (Table 5) haplotypes *A*33:01-DRB1*07:01* and *B*44:03-DRB1*07:01* were significantly increased, and *A*26:01-DRB1*03:01* was significantly reduced in vitiligo cases, in both initial and replication studies.

Four-locus haplotypes were constructed for North Indian samples and a significant increase of *A*33:01-C*07:01-B*44:03-DRB1*07:01* and *A*02:01-C*07:01-B*44:03-DRB1*07:01*, and a significant reduction in haplotype *A*26:01-C*07:02-B*08:01-DRB1*03:01* in vitiligo patients was observed as compared with controls (Supplementary Table S8 online).

HLA class I and II alleles and haplotypes in different manifestations of vitiligo: generalized and localized vitiligo as compared with unaffected controls

To study the association of *MHC* alleles with different manifestations of vitiligo, cases were divided into two clinical types—generalized and localized. *A*33:01*, *B*44:03*, *C*07:01*, and *DRB1*07:01* were significantly increased in

Table 3. *HLA-A, HLA-B,* and *HLA-DRB1* alleles showing significant differences in all vitiligo and generalized and localized vitiligo as compared with healthy controls in *DRB1*07:01*-negative samples in North Indian and Gujarat populations

	Vitiligo	Generalized vitiligo	Localized vitiligo	Controls	Vitiligo versus controls		Generalized vitiligo versus controls		Localized vitiligo versus controls	
HLA allele	No. (%)	No. (%)	No. (%)	No. (%)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
HLA-A										
*A*02:01* N India	92 (13.53)	70 (14.11)	22 (11.96)	68 (10.40)	0.078	1.34 (0.95–1.91)	0.055	1.41 (0.97–2.05)	0.54	1.17 (0.66–1.98)
Gujarat	13 (7.65)	8 (6.84)	5 (9.43)	42 (13.38)	0.058	0.53 (0.25–1.05)	0.059	0.47 (0.18–1.06)	0.29[1]	0.72 (0.37–1.41)[2]
*A*24:02* N India	146 (21.47)	105 (21.17)	41 (22.28)	143 (21.87)	0.86	0.97 (0.74–1.27)	0.77	0.95 (0.71–1.28)	0.90	1.02 (0.67–1.53)
Gujarat	32 (18.82)	23 (19.66)	9 (16.98)	88 (28.03)	0.025	0.59 (0.36–0.95)	0.077	0.62 (0.35–1.07)	0.09	0.52 (0.21–1.15)
*A*26:01* N India	41 (6.03)	32 (6.45)	9 (4.89)	69 (10.55)	0.0027	0.54 (0.35–0.82)	0.015	0.58 (0.36–0.92)	0.019	0.43 (0.18–0.90)
Gujarat	11 (6.47)	8 (6.84)	3 (5.66)	37 (11.78)	0.061	0.51 (0.23–1.07)	0.13	0.54 (0.21–1.24)	0.13[1]	0.51 (0.22–1.14)[2]
*A*31:01* N India	24 (3.53)	17 (3.43)	7 (3.80)	50 (7.65)	0.001	0.44 (0.25–0.74)	0.0025	0.42 (0.22–0.76)	0.06	0.47 (0.18–1.08)
Gujarat	9 (5.29)	7 (5.98)	2 (3.77)	16 (5.10)	0.92	1.04 (0.39–2.56)	0.71	1.18 (0.40–3.14)	0.50[1]	0.87 (0.33–2.29)[2]
*A*33:01* N India	94 (13.82)	59 (11.90)	35 (19.02)	71 (10.86)	0.09	1.31 (0.93–1.85)	0.58	1.10 (0.75–1.62)	0.003	1.92 (1.19–3.05)
Gujarat	20 (11.76)	15 (12.82)	5 (9.43)	34 (10.83)	0.75	1.09 (0.57–2.04)	0.56	1.21 (0.58–2.39)	0.49[1]	0.92 (0.47–1.80)[2]
*A*68:01* N India	107 (15.74)	87 (17.54)	20 (10.87)	100 (15.29)	0.82	1.03 (0.76–1.40)	0.30	1.17 (0.84–1.63)	0.13	0.67 (0.38–1.14)
Gujarat	44 (25.88)	32 (27.35)	12 (22.64)	38 (12.10)	0.0001	2.53 (1.52–4.23)	0.0001	2.73 (1.54–4.79)	0.038	2.12 (0.93–4.57)
HLA-B										
*B*08:01* N India	37 (5.44)	30 (6.05)	7 (3.80)	96 (14.68)	1.80×10^{-8}	0.33 (0.21–0.50)	3.47×10^{-6}	0.37 (023–0.58)	0.00007	0.22 (0.08–0.50)
Gujarat	8 (4.71)	4 (3.45)	4 (3.45)	32 (10.32)	0.033	0.42 (0.16–0.98)	0.014[1]	0.34 (0.16–0.70)[2]	0.35[1]	0.76 (0.37–1.58)[2]
*B*13:01* N India	40 (5.88)	30 (6.05)	10 (5.43)	15 (2.29)	0.00098	2.66 (1.42–5.23)	0.001	2.74 (1.41–5.54)	0.02	2.44 (0.96–5.92)
Gujarat	5 (2.94)	3 (2.59)	2 (1.72)	2 (0.65)	0.057[1]	4.10 (1.41–11.90)[2]	0.12[1]	3.80 (1.19–12.11)[2]	0.10[1]	5.87 (1.67–20.64)[2]
*B*15:02* N India	39 (5.74)	29 (5.85)	10 (5.43)	16 (2.45)	0.002	2.42 (1.30–4.9)	0.003	2.47 (1.28–4.93)	0.038	2.29 (0.91–5.47)
Gujarat	16 (9.41)	13 (11.21)	3 (2.59)	26 (8.39)	0.70	1.13 (0.55–2.27)	0.34	1.39 (0.63–2.94)	0.34[1]	0.72 (0.32–1.65)[2]
*B*37:01* N India	70 (10.29)	53 (10.69)	17 (9.24)	29 (4.43)	0.00004	2.47 (1.55–4.01)	0.00004	2.57 (1.57–4.27)	0.01	2.19 (1.10–4.24)
Gujarat	29 (17.06)	19 (16.38)	10 (8.62)	23 (7.42)	0.001	2.56 (1.37–4.82)	0.0057	2.44 (1.19–4.90)	0.008	2.83 (1.12–6.68)
*B*44:03* N India	39 (5.74)	24 (4.84)	15 (8.15)	18 (2.75)	0.007	2.14 (1.18–4.03)	0.06	1.79 (0.92–3.55)	0.00087	3.13 (1.43–6.73)
Gujarat	4 (2.35)	3 (2.59)	1 (0.8)	14 (4.52)	0.17[1]	0.55 (0.26–1.18)[2]	0.27[1]	0.63 (0.27–1.45)[2]	0.31[1]	0.57 (0.17–1.91)[2]
*B*51:01* N India	93 (13.68)	79 (15.93)	14 (7.61)	138 (21.10)	0.0003	0.59 (0.43–0.79)	0.026	0.70 (0.51–0.97)	0.000027	0.30 (0.16–0.55)
Gujarat	23 (13.53)	16 (13.79)	7 (6.03)	42 (13.55)	0.99	0.99 (0.55–1.77)	0.94	1.02 (0.51–1.95)	0.90	0.95 (0.33–2.30)
*B*57:01* N India	24 (3.53)	17 (3.43)	7 (3.80)	25 (3.82)	0.77	0.92 (0.49–1.69)	0.72	0.89 (0.44–1.74)	0.99	0.99 (0.35–2.41)
Gujarat	8 (4.71)	3 (2.59)	5 (4.31)	22 (7.10)	0.30	0.64 (0.24–1.55)	0.055[1]	0.39 (0.17–0.89)[2]	0.37[1]	1.42 (0.71–2.84)[2]
*B*58:01* N India	39 (5.74)	24 (4.84)	15 (8.15)	59 (9.02)	0.02	0.61 (0.39–0.95)	0.006	0.51 (0.30–0.85)	0.71	0.89 (0.45–1.64)
Gujarat	7 (4.12)	3 (2.59)	4 (3.45)	16 (5.16)	0.60	0.78 (0.26–2.08)	0.19[1]	0.55 (0.24–1.26)[2]	0.34[1]	1.59 (0.74–3.42)[2]
HLA-DRB1										
*DRB1*01:01* N India	35 (5.15)	25 (5.04)	10 (5.43)	35 (5.35)	0.86	0.95 (0.57–1.60)	0.81	0.93 (0.53–1.63)	0.96	1.01 (0.44–2.15)
Gujarat	4 (2.34)	2 (1.71)	2 (3.70)	25 (7.58)	0.01[1]	0.32 (0.15–0.66)[2]	0.01[1]	0.25 (0.10–0.65)[2]	0.23[1]	0.57 (0.22–1.46)[2]
*DRB1*03:01* N India	65 (9.56)	49 (9.88)	16 (8.70)	155 (23.70)	3.46×10^{-12}	0.34 (0.24–0.46)	1.22×10^{-9}	0.35 (0.24–0.50)	8.13×10^{-6}	0.30 (0.16–0.53)
Gujarat	12 (7.02)	7 (5.98)	5 (9.26)	60 (18.18)	0.0007	0.33 (0.16–0.66)	0.0015	0.28 (0.10–0.65)	0.071[1]	0.49 (0.26–0.95)[2]
*DRB1*04:03* N India	65 (9.56)	46 (9.27)	19 (10.33)	47 (7.19)	0.11	1.36 (0.90–2.06)	0.19	1.32 (0.84–2.06)[2]	0.16	1.48 (0.80–2.66)
Gujarat	2 (1.17)	0 (0.00)	2 (3.70)	24 (7.27)	0.0016[1]	0.18 (0.07–0.46)[2]	0.0005[1]	0.05 (0.007–0.38)[2]	0.26[1]	0.59 (0.23–1.52)[2]
*DRB1*10:01* N India	105 (15.44)	81 (16.33)	24 (13.04)	64 (9.79)	0.0019	1.68 (1.19–2.38)	0.0009	1.79 (1.24–2.59)	0.20	1.38 (0.80–2.32)
Gujarat	40 (23.39)	26 (22.22)	14 (25.93)	39 (11.82)	0.0007	2.27 (1.35–3.81)	0.006	2.13 (1.17–3.81)	0.005	2.61 (1.19–5.43)
*DRB1*11:01* N India	73 (10.74)	56 (11.29)	17 (9.24)	77 (11.77)	0.55	0.90 (0.63–1.28)	0.79	0.95 (0.64–1.39)	0.33	0.76 (0.41–1.34)
Gujarat	20 (11.70)	17 (14.53)	3 (5.56)	41 (12.42)	0.81	0.93 (0.49–1.69)	0.56	1.19 (0.60–2.27)	0.10[1]	0.47 (0.21–1.05)[2]
*DRB1*12:02* N India	52 (7.65)	41 (8.27)	11 (5.98)	12 (1.83)	6.84×10^{-7}	4.42 (2.30–9.19)	2.58×10^{-7}	4.82 (2.44–10.17)	0.002	3.40 (1.33–8.56)

Table 3 continued on following page

Table 3. Continued

	Vitiligo	Generalized vitiligo	Localized vitiligo	Controls	Vitiligo versus controls		Generalized vitiligo versus controls		Localized vitiligo versus controls	
HLA allele	No. (%)	No. (%)	No. (%)	No. (%)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
Gujarat	12 (7.02)	10 (8.55)	2 (3.70)	22 (6.67)	0.88	1.05 (0.46–2.29)	0.49	1.30 (0.53–2.98)	0.31[1]	0.65 (0.25–1.68)[2]
*DRB1*14:04* N India	92 (13.53)	62 (12.50)	30 (16.30)	56 (8.56)	0.0039	1.67 (1.16–2.41)	0.029	1.52 (1.02–2.27)	0.002	2.08 (1.24–3.42)
Gujarat	22 (12.87)	16 (13.68)	6 (11.11)	39 (11.82)	0.73	1.10 (0.59–1.98)	0.13	1.51 (0.83–2.68)	0.88	0.93 (0.30–2.38)

Abbreviations: CI, confidence interval; N, North; OR, odds ratio.
[1]*P*-value was calculated by using Fisher's exact test.
[2]OR was calculated by using Woolf's method with Haldane's modification.
All patient and control samples with *DRB1*07:01* allele were removed to study the contribution of alleles independent of *DRB1*07:01* in susceptibility to vitiligo. All vitiligo (*N*=680), generalized (*N*=496), and localized (*N*=184) vitiligo samples from North India were compared with healthy controls (*N*=654) for *HLA-A*, *HLA-B*, and *HLA-DRB1* loci. From Gujarat, all vitiligo (*N*=170), generalized (*N*=117), and localized (*N*=53) vitiligo samples were compared with 314 unaffected controls for *HLA-A* locus. Overall, 170 vitiligo samples, 116 generalized and 54 localized, were compared with 314 samples from unaffected controls for *HLA-B* locus, and 171 vitiligo samples, 117 generalized and 54 localized, were compared with 330 unaffected controls for *HLA-DRB1* locus.

the initial study in both generalized and localized vitiligo. However, only *B*44:03* and *DRB1*07:01* were associated in the replication study with both the generalized and localized types of vitiligo (Table 2 and Supplementary Table S1 online). Alleles associated with different manifestations of vitiligo were *HLA-A*02:01*, *B*37:01*, *B*57:01*, *DRB1*04:03*, and *DRB1*10:01*, which were significantly increased, and *A*31:01*, *B*58:01*, *C*04:01*, *DRB1*01:01*, *DRB1*11:01*, and *DRB1*15:02* were significantly reduced in generalized vitiligo cases and not in localized vitiligo cases from North India. *HLA-A*30:01*, *A*33:01*, and *A*68:01* were significantly increased, and *B*58:01* and *DRB1*01:01* were significantly reduced in generalized vitiligo cases and not in localized vitiligo cases from Gujarat as compared with unaffected controls.

In the absence of *DRB1*07:01*, *B*37:01* was significantly increased in both North Indian and Gujarati patients, irrespective of the type of vitiligo. In addition, *A*68:01* and *DRB1*10:01* were significantly increased in patients from Gujarat and *B*13:01*, *B*15:02*, *DRB1*12:02*, and *DRB1*14:04* were significantly increased in North Indian patients, irrespective of the type of vitiligo. *A*33:01* and *B*44:03* were increased in localized cases and *DRB1*10:01* was increased in generalized cases from North India (Table 3).

Haplotype *A*33:01-B*44:03-DRB1*07:01* (Table 4) was significantly increased in both generalized and localized cases in the initial study and only in generalized cases in the replication study. *A*24:02-B*44:03-DRB1*07:01* was significantly increased in generalized cases from North India, whereas both generalized and localized cases from Gujarat showed a significant increase in the frequency of this haplotype when compared with unaffected controls. Haplotype *A*02:01-B*44:03-DRB1*07:01* was significantly increased in both generalized and localized vitiligo cases from North India.

Two-locus haplotype analysis showed that *B*44:03-DRB1*07:01* was significantly increased in both generalized and localized cases in both initial and replication studies (Table 5). *A*33:01-DRB1*07:01* and *A*02:01-DRB1*07:01* were significantly increased in both generalized and localized vitiligo cases in the initial study; however, only haplotype *A*33:01-DRB1*07:01* was significantly increased in generalized vitiligo cases from Gujarat. Haplotype *A*26:01-DRB1*03:01* was significantly reduced in both generalized and localized cases from North India and Gujarat, although *P*-value did not reach statistical significance for localized cases from Gujarat because of smaller sample size. *B*08:01-DRB1*03:01* was significantly reduced in both generalized and localized vitiligo cases from North India and Gujarat.

Specific amino-acid signatures of HLA peptide-binding pockets predispose to vitiligo

Amino-acid signatures of the peptide-binding pockets of HLA-A, -B, and -DRB1 were compared in vitiligo cases with unaffected controls (Figure 1 and Supplementary Tables S9 and S10 online). For HLA-A and -B, five key residues constituting the B pocket (positions 9, 45, 63, 66, and 67) and four key residues constituting the F pocket (positions 77, 80, 81, and 116) of the α1 and α2 domains of the α-chain were investigated (Sidney *et al.*, 2008). For DRB1, 11 residues representing integral parts of peptide-binding pockets of DR β-chain were analyzed at positions 26, 28, 30, 37, 47, 67, 70, 71, 74, 77, and 86 (Menconi *et al.*, 2010). The protein sequences were downloaded from the HLA database (http://www.ebi.ac.uk/imgt/hla/align.html), and the amino acids present at the aforesaid positions were compared in patients and controls. Approximately 8–10% of the samples were homozygous for one or more loci in patients and controls. Thus, to avoid overrepresentation of certain amino acids at particular positions because of homozygosity, we considered phenotype frequencies of the alleles; i.e., the homozygous samples were counted as having only one allele. The results show that that $Glu^{28\beta}$, $Leu^{30\beta}$/$Arg^{30\beta}$ $Phe^{37\beta}$, $Tyr^{47\beta}$, $Ile^{67\beta}$, $Asp^{70\beta}$, $Arg^{71\beta}$, $Gln^{74\beta}$, $Thr^{77\beta}$, and $Gly^{86\beta}$ make the molecular signature of the peptide-binding pockets of DRB1 for predisposition to develop vitiligo in both the initial and replication studies (Figure 1). $Asp^{28\beta}$, $Tyr^{30\beta}$/$Cys^{30\beta}$, $Ser^{37\beta}$, $Phe^{47\beta}$, $Leu^{67\beta}$, $Gln^{70\beta}$, $Lys^{71\beta}$, Ala/$Arg^{74\beta}$, $Asn^{77\beta}$, and $Val^{86\beta}$

Table 4. *HLA-A-B-DRB1* (three-locus) haplotypes significantly different in vitiligo cases as compared with controls from North India and Gujarat

		North India vitiligo versus North India controls			Gujarat vitiligo versus Gujarat controls		
HLA haplotype	Sample type	No. (%)	*P*-value	OR (95% CI)	No. (%)	*P*-value	OR (95% CI)
*A*33:01-B*44:03-DRB1*07:01*	Controls	27 (2.99)			14 (3.57)		
	All cases	226 (16.09)	8.75×10^{-23}	6.21 (4.01–9.72)	42 (11.93)	0.000016	3.65 (1.91–7.37)
	Generalized	189 (17.22)	1.94×10^{-24}	6.74 (4.43–10.60)	34 (13.70)	2.09×10^{-6}	4.28 (2.18–8.83)
	Localized	37 (12.05)	9.18×10^{-10}	4.44 (2.57–7.72)	8 (7.69)	0.07	2.25 (0.79–5.93)
*A*02:01-B*44:03-DRB1*07:01*	Controls	0 (0.00)			0 (0.00)		
	All cases	33 (2.35)	6.66×10^{-8} [1]	44.08 (6.11–317.86)[2]	1 (0.28)	0.47[1]	3.34 (0.34–32.27)[2]
	Generalized	29 (2.64)	2.33×10^{-8} [1]	49.83 (6.83–360.03)[2]	0 (0.00)		
	Localized	4 (1.30)	0.004[1]	26.76 (3.38–211.67)[2]	1 (0.96)	0.20[1]	11.37 (1.17–109.93)[2]
*A*24:02-B*44:03-DRB1*07:01*	Controls	4 (0.44)			2 (0.51)		
	All cases	26 (1.85)	0.0019[1]	3.83 (1.88–7.80)[2]	12 (3.40)	0.0034[1]	5.73 (2.18–15.06)[2]
	Generalized	23 (2.09)	0.0009[1]	4.37 (2.13–8.93)[2]	7 (2.82)	0.020[1]	4.85 (1.75–13.43)[2]
	Localized	3 (0.97)	0.25[1]	2.29 (0.85–6.18)[2]	5 (4.80)	0.005[1]	8.63 (2.96–25.13)[2]
*A*26:01-B*08:01-DRB1*03:01*	Controls	37 (4.10)			17 (4.33)		
	All cases	20 (1.42)	0.00005	0.33 (0.18–0.60)	2 (0.56)	0.0007[1]	0.15 (0.05–0.39)[2]
	Generalized	16 (1.45)	0.00025	0.34 (0.18–0.64)	1 (0.40)	0.0016[1]	0.13 (0.04–0.42)[2]
	Localized	4 (1.30)	0.010[1]	0.34 (0.17–0.68)[2]	1 (0.96)	0.08[1]	0.31 (0.09–1.02)[2]
*A*01:01-B*57:01-DRB1*07:01*	Controls	30 (3.32)			27 (6.88)		
	All cases	81 (5.76)	0.0075	1.77 (1.42–2.82)	27 (7.67)	0.68	1.12 (0.61–2.03)
	Generalized	65 (5.92)	0.0065	1.83 (1.15–2.95)	22 (8.87)	0.35	1.31 (0.70–2.46)
	Localized	16 (5.21)	0.13	1.59 (0.80–3.08)	5 (4.80)	0.30[1]	0.73 (0.37–1.43)[2]
*A*11:01-B*44:03-DRB1*07:01*	Controls	16 (1.77)			1 (0.25)		
	All cases	43 (3.06)	0.055	1.74 (0.95–3.34)	9 (2.55)	0.006[1]	7.21 (2.12–24.49)[2]
	Generalized	36 (3.28)	0.035	1.87 (1.00–3.65)	7 (2.82)	0.006[1]	8.1 (2.33–28.14)[2]
	Localized	7 (2.28)	0.57	1.29 (0.44–3.36)	2 (1.92)	0.11[1]	6.36 (1.50–26.85)[2]
*A*11:01-B*57:01-DRB1*07:01*	Controls	8 (0.99)			15 (3.82)		
	All cases	14 (0.99)	0.79	1.12 (0.43–3.10)	5 (1.42)	0.034[1]	0.38 (0.19–0.77)[2]
	Generalized	12 (1.09)	0.64	1.23 (0.46–3.50)	3 (1.20)	0.04[1]	0.34 (0.15–0.79)[2]
	Localized	2 (0.65)	0.51[1]	1.69 (0.54–4.91)[2]	2 (1.92)	0.27[1]	0.59 (0.22–1.54)[2]

Abbreviations: CI, confidence interval; OR, odds ratio.
[1]*P*-value was calculated by using Fisher's exact test.
[2]OR was calculated by using Woolf's method with Haldane's modification.
Three-locus haplotypes were constructed using Arlequin ver 3.5 for 1,404 vitiligo patients referred to as all cases in the table (1,097 generalized and 307 localized vitiligo cases) from North India and 352 vitiligo patients from Gujarat (248 generalized and 104 localized vitiligo cases) and compared with 902 and 392 unaffected controls from North India and Gujarat, respectively.

make the molecular signature of DRB1 for the protection from vitiligo in both North India and Gujarat. The molecular signature for HLA-A alleles for predisposition to develop the disease is actually specific for HLA-A allele *A*33:01*, which is in LD with *DRB1*07:01*. For HLA-B locus, $Lys^{45\alpha}$, $Glu^{63\alpha}$, $Ser^{67\alpha}$, $Asn^{77\alpha}$, $Thr^{80\alpha}$, $Ala^{81\alpha}$, and $Ser^{99\alpha}$ makes the molecular signature for predisposition to develop vitiligo, and $Thr^{45\alpha}$, $Asn^{63\alpha}$, $Phe^{67\alpha}$, $Ser^{77\alpha}$, $Asn^{80\alpha}$, $Leu^{81\alpha}$, and $Tyr^{99\alpha}$ makes the molecular signature for protection from vitiligo in both the initial and replication studes. In addition, significant increase of $Met^{45\alpha}$, $Cys^{67\alpha}$, $Asp^{77\alpha}$, and $Asp^{116\alpha}$ in North Indian samples suggests the involvement of alleles other than

Table 5. *HLA-A-DRB1* and *HLA-B-DRB1* (two-locus) haplotypes showing significant differences in vitiligo patients as compared with controls from North India and Gujarat

		North India vitiligo versus North India controls			Gujarat vitiligo versus Gujarat controls		
HLA haplotype	Sample type	No. (%)	*P*-value	OR (95% CI)	No. (%)	*P*-value	OR (95% CI)
*A*33:01-DRB1*07:01*	Controls	29 (7.39)			15 (3.82)		
	All cases	251 (17.87)	6.97×10^{-26}	6.55 (4.39–10.08)	44 (12.50)	0.00001	3.59 (1.91–7.07)
	Generalized	212 (19.32)	3.50×10^{-28}	7.21 (4.81–11.14)	36 (14.51)	1.44×10^{-6}	4.26 (2.21–8.57)
	Localized	39 (12.70)	4.58×10^{-10}	4.38 (2.58–7.48)	8 (7.69)	0.09	2.09 (0.74–5.43)
*A*02:01-DRB1*07:01*	Controls	7 (1.10)			0 (0.00)		
	All cases	66 (4.70)	1.49×10^{-7}	6.30 (2.87–16.35)	3 (0.85)	0.10[1]	7.86 (0.96–64.05)[2]
	Generalized	56 (5.10)	3.53×10^{-8}	6.87 (3.10–17.96)	0 (0.00)		
	Localized	10 (3.25)	0.001	4.30 (1.46–13.43)	3 (2.88)	0.009[1]	27.06 (3.31–221.26)[2]
*B*44:03-DRB1*07:01*	Controls	82 (9.09)			19 (4.84)		
	All cases	402 (28.63)	2.43×10^{-29}	4.01 (3.09–5.23)	86 (24.43)	1.84×10^{-14}	6.34 (3.71–11.30)
	Generalized	343 (31.26)	1.74×10^{-33}	4.54 (3.48–5.97)	63 (25.40)	3.45×10^{-14}	6.68 (3.80–12.15)
	Localized	59 (19.21)	1.79×10^{-6}	2.37 (1.62–3.47)	23 (22.11)	1.87×10^{-8}	5.57 (2.74–11.33)
*B*57:01-DRB1*07:01*	Controls	47 (5.21)			45 (4.98)		
	All cases	128 (9.11)	0.0005	1.82 (1.28–2.63)	44 (12.50)	0.66	1.10 (0.69–1.75)
	Generalized	103 (9.38)	0.0004	1.88 (1.30–2.75)	35 (14.11)	0.32	1.26 (0.76–2.08)
	Localized	25 (8.14)	0.60	1.61 (0.93–2.73)	9 (8.65)	0.41	0.73 (0.30–1.58)
*A*24:02-DRB1*12:02*	Controls	3 (0.33)			13 (3.31)		
	All cases	12 (0.85)	0.10[1]	2.30 (0.99–5.34)[2]	3 (0.85)	0.01[1]	0.28 (0.12–0.65)[2]
	Generalized	8 (0.72)	0.18[1]	2.00 (0.82–4.84)[2]	3 (1.20)	0.076[1]	0.40 (0.17–0.92)[2]
	Localized	4 (1.30)	0.07[1]	3.81 (1.41–10.27)[2]	0 (0.00)	0.045[1]	0.13 (0.02–0.99)[2]
*A*26:01-DRB1*03:01*	Controls	37 (4.10)			17 (4.33)		
	All cases	20 (1.42)	0.00005	0.33 (0.18–0.60)	2 (0.56)	0.0007[1]	0.15 (0.05–0.39)[2]
	Generalized	16 (1.45)	0.00025	0.34 (0.18–0.64)	1 (0.40)	0.0016[1]	0.13 (0.04–0.42)[2]
	Localized	4 (1.30)	0.01[1]	0.34 (0.17–0.69)[2]	1 (0.96)	0.07[1]	0.31 (0.09–1.02)[2]
*B*08:01-DRB1*03:01*	Controls	71 (7.87)			27 (6.88)		
	All cases	24 (1.70)	3.70×10^{-13}	0.20 (0.12–0.33)	4 (1.61)	0.00004[1]	0.17 (0.08–0.34)[2]
	Generalized	18 (1.64)	1.80×10^{-11}	0.19 (0.11–0.33)	3 (1.20)	0.0004[1]	0.18 (0.08–0.42)[2]
	Localized	6 (1.95)	0.0002	0.23 (0.08–0.54)	1 (0.96)	0.01[1]	0.19 (0.06–0.62)[2]

Abbreviations: CI, confidence interval; OR, odds ratio.
[1]*P*-value was calculated by using Fisher's exact test.
[2]OR was calculated by using Woolf's method with Haldane's modification.
Two-locus haplotypes (*HLA-A-HLA-DRB1* and *HLA-B-HLA-DRB1*) were deduced from three-locus haplotypes (Arlequin ver 3.5) for patients and controls as described in Table 4.

the predisposing alleles, which may be in low frequencies (Figure 1, Supplementary Table S10 online).

Subtle differences were observed in the molecular signatures of the peptide-binding pockets of DR β-chain in localized and generalized vitiligo. Interestingly, localized vitiligo patients show similarities in the amino-acid signatures with generalized vitiligo at positions $Glu^{28\beta}$, $Leu^{30\beta}$, $Phe^{37\beta}$, $Tyr^{47\beta}$, and $Gln^{74\beta}$ in both the initial and replication studies. However, they show similarities with unaffected controls at positions $Arg^{30\beta}$/$Cys^{30\beta}$, $Ser^{37\beta}$, $Ile^{67\beta}$/$Leu^{67\beta}$ $Asp^{70\beta}$, and

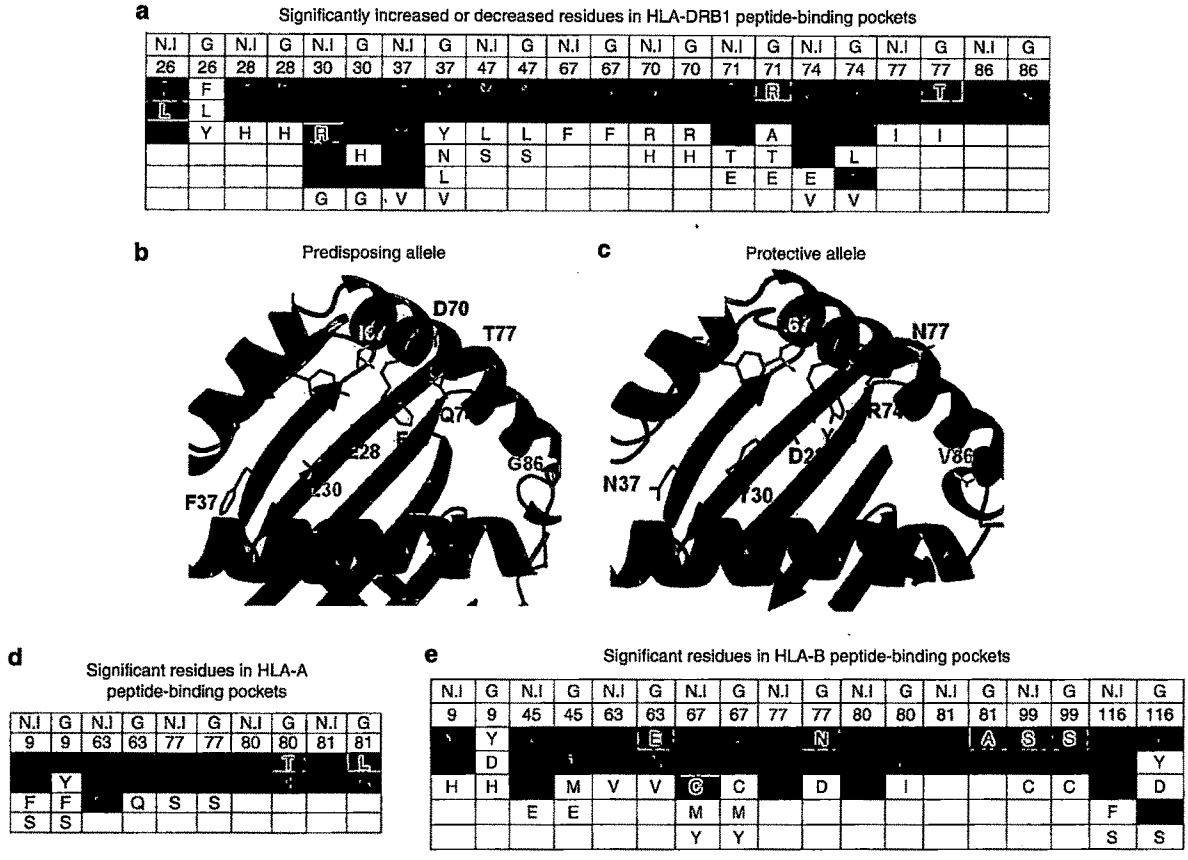

Figure 1. Amino-acid signatures for the peptide-binding pockets of HLA molecules. (**a**) For DR β1-chain in vitiligo patients from North India and Gujarat. Amino acids shown in red are significantly increased in vitiligo patients; amino acids shown in green are significantly reduced in vitiligo patients as compared with controls. More than one amino acid at certain positions being red or green suggests the involvement of multiples alleles. (**b**) Ribbon diagram for peptide-binding clefts for predisposing and (**c**) protective DRB1 alleles (using PyMOL software, PyMOL Molecular Graphics System, Schrödinger, San Carlos, CA). (**d**) Amino-acid signature for HLA-A locus. (**e**) Amino-acid signature for HLA-B locus in vitiligo. ■ Significantly increased $P<0.009$ to 10^{-29}; ▣ Significantly increased $P<0.05$–0.01; ■ Significantly reduced $P<0.009$ to 10^{-28}; ■ Significantly reduced $P<0.05$–0.01; □ Nonsignificant.

$Ala^{74\beta}$ in North Indians, and $Cys^{30\beta}$, $Ser^{37\beta}$, $Asp^{70\beta}$, $Arg^{71\beta}$/Lys $Ser^{71\beta}$, $Ala^{74\beta}$/$Arg^{74\beta}$, and $Thr^{77\beta}$/$Asn^{77\beta}$ in Gujaratis (data not shown).

DISCUSSION

Our results show that *A*33:01*, *B*44:03*, and *DRB1*07:01* were significantly increased in both initial and replication studies, suggesting that these alleles are the markers for vitiligo in both North India and Gujarat. Association of *DRB1*07:01* with vitiligo seems to be primary, because in the absence of *A*33:01*, *B*44:03*, or *B*37:01*, *DRB1*07:01* still remains highly significant in both the populations studied, whereas the converse was not true. The basic predisposing alleles in both localized and generalized vitiligo are the same; however, similarities with the controls in terms of allele frequencies of some alleles and amino-acid signature of the DR β-chain seem to be protective from generalized distribution of the lesions in localized vitiligo (Supplementary Table S11 online).

Haplotype analysis showed the presence of 177 and 68 *DRB1*07:01* haplotypes in North Indian and Gujarati cases, respectively, suggesting that enough recombinational events have taken place during the course of evolution, giving rise to such a diversity of haplotypes; however, some of the predisposing and protective haplotypes are still conserved. The haplotype *A*33:01-B*44:03-DRB1*07:01* was significantly increased in generalized and localized vitiligo cases from North India and only generalized cases from Gujarat. Although the localized cases from Gujarat showed an OR of 2.25 for this haplotype, the *P*-value was not significant, probably because of smaller sample size for the localized cases from Gujarat.

Very few studies have been conducted on association of HLA alleles with vitiligo using low-resolution molecular methods and still fewer using high-resolution typing methods. A study on 41 Turkish vitiligo cases showed *DRB1*03*, *DRB1*04*, and *DRB1*07* to be increased in the vitiligo cases (Tastan *et al.*, 2004). However, we observed *DRB1*07:01* to

be positively associated and *DRB1*03:01* to be negatively associated with vitiligo in both initial and replication studies. Presence of *DRB1*07:01* has been reported in patients from Slovakia (Buc *et al.*, 1998) and China (Ren *et al.*, 2009), whereas HLA class I alleles have not been reported in these studies. A meta-analysis of 11 case-control studies ($N=777$) showed that class I allele HLA-A2 is associated with vitiligo (Liu *et al.*, 2007). HLA-A2 is a cluster of 266 alleles, one of which, *HLA-A*02:01*, was significantly increased in generalized vitiligo cases ($P<0.001$, OR = 1.57) from North India, although only 14.59% of the generalized vitiligo patients and 9.76% of unaffected controls had this allele (Table 2). The haplotype *A*02:01-B*44:03-DRB1*07:01* was significantly increased in both generalized (2.64%) and localized cases (1.3%) from North India as compared with controls who lacked this haplotype altogether. However, our data suggest that the primary association of *HLA* with vitiligo in North India and Gujarat is not with *A*02:01*, but with A*33:01, *B*44:03*, and *DRB1*07:01*.

Analysis of two-locus haplotypes for *HLA-A-DRB1* and *HLA-B-DRB1* suggests that the haplotype *B*44:03-DRB1*07:01* is the marker for both generalized and localized vitiligo in both the populations studied. *DRB1*07:01* was also in LD with *A*02:01*, *A*33:01*, and *B*57:01*. These data suggest that the autoreactive $CD4^+$ T-helper cells are restricted by *HLA-DRB1*07:01* and the autoreactive $CD8^+$ cytotoxic T cells may be restricted by *HLA-A*33:01*, *A*02:01*, *B*44:03*, and *B*57:01* in the Indian populations studied.

Association of *MHC* alleles with a disease gains importance because of the antigen-presenting function of the MHC. The peptides presented by the MHC molecules have allele-specific motifs (Falk *et al.*, 1991). The affinity of the peptide to a particular MHC molecule is determined by the amino-acid residues present in peptide-binding pockets of the peptide-binding groove. Shared amino acids in the peptide-binding pockets have been demonstrated in autoimmune diseases such as Type 1 diabetes (Todd *et al.*, 1987), rheumatoid arthritis (Winchester, 1994), and thyroiditis (Menconi *et al.*, 2010). Investigation of amino-acid signatures for the peptide-binding pockets of HLA-A and HLA-B α-chain and HLA-DR β-chain revealed specific molecular signatures for predisposition and protection from the disease. These results suggest affinity of autoantigenic peptides for predisposing MHC class II and class I molecules that may be involved in orchestrating (through $CD4^+$ T cells) and implementing the autoimmune responses (through $CD8^+$ T cells) in vitiligo, respectively.

Generalized vitiligo is considered to be an autoimmune disease, whereas localized vitiligo has not been considered an autoimmune disease. We observed *B*44:03* and *DRB1*07:01* to be predisposing in both generalized and localized vitiligo patients in both the populations studied, beside some differences in the frequencies of some other alleles. To our knowledge, this is previously unreported. Association of *MHC* alleles with localized vitiligo clearly shows that there is a need to relook at the etiopathogenesis of localized vitiligo. Although it may be an autoimmune disorder, similarities with unaffected controls in terms of *HLA* alleles and amino-acid signatures of the peptide-binding pockets of DR β-chain may be contributing to the localized distribution of the lesions.

MATERIALS AND METHODS

Patient group

DNAs were extracted from 1,404 North Indian patients with vitiligo enrolled at Dr Ram Manohar Lohia Hospital and All India Institute of Medical Sciences, New Delhi, after obtaining informed consent. These subjects belonged to North Indian states of Uttar Pradesh, Himachal Pradesh, Bihar, Haryana, Punjab, and Delhi. All cases and control samples were collected after Institutional Human Ethics Committee's clearance from all the institutes/hospitals involved, following the Declaration of Helsinki Principles. Diagnosis of vitiligo was based on clinical examination done by dermatologists. Clinically, the cases were classified as having generalized (vulgaris, acrofacial, and universalis) or localized (focal, acral, mucosal, and segmental) forms of vitiligo. The replication study was carried out on 355 vitiligo cases from Gujarat, a state in west of India (Table 1).

It may be pertinent to mention here that the two groups studied in the initial and replication studies form two independent endogamous groups that do not inter-marry.

Unaffected controls

A total of 902 ethnically matched unaffected controls from North India were used in the initial study and 441 ethnically matched unaffected controls from Gujarat were studied in the replication study. None of the unaffected controls had personal or family history of vitiligo or any other autoimmune disease. Of the 902 controls from North India, blood samples of 308 were collected at National Institute of Immunology, Dr Ram Manohar Lohia Hospital and All India Institute of Medical Sciences, New Delhi, and the remaining 594 samples were procured from the Institute of Genomics and Integrative Biology, New Delhi, which were collected from the aforesaid states of North India as a part of the Indian Genome Variation program, and the details of the sample collection have been explained elsewhere (Consortium, 2005).

Study of *HLA-A*, *HLA-B*, *HLA-C*, and *HLA-DRB1* alleles

DNA extraction was carried out by standard procedures from blood samples collected in anticoagulant EDTA. Alleles of *HLA-A*, *HLA-B*, *HLA-C*, and *HLA-DRB1* were studied using PCR, followed by hybridization with sequence-specific oligonucleotide probes as described earlier (Israni *et al.*, 2009), using a bead-based technology (Luminex, Austin, TX) following the manufacturer's instructions (Labtype SSO kit from One Lambda, Canoga Park, LA). All North Indian samples were typed for *HLA-A*, *HLA-B*, and *HLA-DRB1* loci. A total of 404 vitiligo and 438 unaffected control samples from North India were typed for HLA-C locus. Of the 355 Gujarat cases, 354, 353, and 355 cases were typed for *HLA-A*, *HLA-B*, and *HLA-DRB1* loci, respectively, and of the 441 unaffected controls from Gujarat population, 408, 407, and 441 individuals were typed for three loci, respectively. Differences in the sample numbers studied for different loci are because of amplification failures in some samples. The latest nomenclature for the *HLA* system was used to designate the alleles of the three loci studied (Marsh *et al.*, 2010).

Haplotype analysis

Estimation of *HLA* haplotype frequencies was carried out using the expectation maximization algorithm (Excoffier and Slatkin, 1995; Excoffier *et al.*, 2005). Arlequin Ver 3.5 (Excoffier *et al.*, 2005) was run using the following settings: expectation maximization algorithm performed at the haplotype level, $\varepsilon = 1e{-}7$, five significant digits for output, 50 starting points for expectation maximization algorithm, and a maximum of 1,000 iterations (Bettencourt *et al.*, 2008) at http://cmpg.unibe.ch/software/arlequin35/. Three- and four-locus haplotypes were thus constructed for both patient and controls samples to identify predisposing and protective haplotypes in the two populations. Two-locus haplotypes were derived from three-locus haplotypes.

Statistical analysis

Vitiligo samples were compared with their respective unaffected controls using χ^2-analysis using Stata 9.2 statistical program. Fisher's exact test was used when the numbers were five or less in any group; i.e., in cases or controls for any allele. In such cases, ORs were calculated using Woolf's method (Woolf, 1955) with Haldane (1956) modification as described earlier (Rani *et al.*, 1998).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Interleukin-4 genetic variants correlate with its transcript and protein levels in patients with vitiligo

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Conflicts of interest
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Summary

Background Vitiligo is an acquired pigmentary disorder resulting from loss of melanocytes. Interleukin (IL)-4 has been shown to stimulate B-cell proliferation, to regulate immunoglobulin class switching (IgG1 and IgE) and to promote T-cell development. Polymorphisms in the IL4 gene are known to increase its expression, thereby implicating its role in vitiligo susceptibility.

Objectives To explore intron 3 VNTR (IVS3) and -590 C/T (rs2243250) promoter polymorphisms in the IL4 gene and to correlate them with the IL4 transcript, serum IL-4 and IgE levels to achieve genotype–phenotype correlation in patients with vitiligo from Gujarat. A replication study was done in a North Indian population.

Methods The case–control study was performed to investigate these polymorphisms in 505 patients and 744 controls in Gujarat, and 596 patients and 397 controls in North India by polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism analysis. IL4 transcript levels were monitored by real-time PCR. Serum IL-4 and IgE levels were measured by enzyme-linked immunosorbent assay and electrochemiluminescence immunoassay, respectively.

Results The genotype frequencies differed significantly between patients with generalized vitiligo and controls for both the polymorphisms in both populations. Allele frequencies significantly differed between patients with generalized vitiligo and controls for both the polymorphisms in the population from Gujarat. Interestingly, genotype and allele frequencies for -590 C/T single nucleotide polymorphism were significantly different between patients with localized vitiligo and controls in both the populations. The study revealed significantly increased IL4 mRNA, serum IL-4 and IgE levels in patients from Gujarat. Age of onset analysis of disease in patients suggested that the TTR2R2, TTR1R2 and CTR2R2 haplotypes had a profound effect in the early onset of the disease.

Conclusions Our results suggest that these polymorphisms of the IL4 gene may be genetic risk factors for susceptibility towards vitiligo and the upregulation of the IL4 transcript, protein and IgE levels in individuals with susceptible haplotypes reveal the crucial role of IL-4 in the pathogenesis of vitiligo.

Vitiligo is a skin disorder with progressive depigmentation of the skin.[1] Absence of melanocytes from the lesional skin due to their destruction has been suggested to be the key event in the pathogenesis of vitiligo.[2] The abnormal immune response frequently observed in patients with vitiligo has led to the suggestion that the condition has an autoimmune component.[3] The autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity.[4,5]

Recently, a number of genes that play a role in vitiligo susceptibility, including *HLA* (human leucocyte antigen), PTPN22 (protein tyrosine phosphatase, nonreceptor type 22), NLRP1 (previously NALP1; NLR family, pyrin domain containing 1), XBP1 (X-box binding protein 1), FOXP1 (forkhead box P1), *IL2RA* [interleukin (IL)-2 receptor, α] have been tested for genetic association with vitiligo.[6]

Cytokines, including interleukins, are important mediators of immunity; hence any imbalance or deficiency in the cytokine

network may lead to autoimmune diseases. Studies indicate that cytokine gene expression is influenced by genetic polymorphisms and these variations appear to be linked with the progression of disease.[7,8] In particular, polymorphisms in the *IL4* gene may have a significant impact on the host immune response. IL-4 is a pleiotropic immunomodulatory cytokine secreted by T-helper (Th) 2 lymphocytes, eosinophils and mast cells.[9] The biological actions of IL-4 include stimulation of IgE and mast cell eosinophil-mediated reactions; IL-4 is the principal cytokine that stimulates B-cell immunoglobulin heavy-chain switching to the IgE isotype, which is the principal mediator of immediate hypersensitivity reactions. Also, IL-4 stimulates the development of Th2 cells from naive CD4+ T cells and functions as an autocrine growth factor for differentiated Th2 cells and also contributes to the maintenance of the Th2 lymphocyte profile that leads to the elevation of baseline IgE levels.[10,11] 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.[12,13] In particular, *IL4* seems to be an attractive candidate gene on the basis of its key role in IgE production and in the induction of inflammation, thereby contributing towards autoimmunity.

Polymorphisms in the *IL4* gene may cause alterations in its levels, leading to a disturbance in immune functioning, thereby implicating its role in several autoimmune diseases including vitiligo. Genetic variants of the promoter region of *IL4* have been related to elevated levels of serum IgE.[14] The *IL4* promoter region possesses a C to T transition at the -590 position. This polymorphism has been shown to be associated with enhanced promoter strength with increased binding of nuclear transcription factors to the promoter leading to different levels of IL-4 and increased IgG levels against specific antigens.[15,16] Another polymorphism frequently described in the *IL4* gene is a 70-bp VNTR (variable number tandem repeat), located in the intron 3 region. The three tandem repeat allele is known to be a high producer of IL-4.[17]

As IL-4 appears to be an important regulator of the immune response, it is of interest to investigate the role of IL-4 in vitiligo. Hence, the aims of this study were: (i) to determine whether the two well-characterized *IL4* polymorphisms, i.e. IVS3 and -590 C/T single nucleotide polymorphism (SNP), are associated with vitiligo susceptibility in populations from Gujarat and North India; (ii) to measure and compare *IL4* mRNA levels in Gujarati patients with vitiligo and healthy controls with different haplotypes; and (iii) to measure and compare serum IL-4 and IgE levels in Gujarati patients with vitiligo and unaffected controls with different haplotypes.

Materials and methods

Study population

The study population included 505 patients with vitiligo (395 generalized and 110 localized vitiligo cases) from Gujarat and 596 patients with vitiligo (464 generalized and 132 localized vitiligo cases) from North India (Table S1; see Supporting information). A total of 744 ethnically and sex-matched unaffected individuals from Gujarat and 397 individuals from North India were included in the study (Table S1). None of the healthy individuals had any evidence of vitiligo or any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India and the All India Institute of Medical Sciences and National Institute of Immunology, New Delhi, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Determination of *IL4* gene polymorphisms

Genomic DNA was extracted from whole blood using a whole blood DNA extraction kit (Bangalore Genei, Bangalore, India). Polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (PCR-RFLP) analyses were used to genotype -590 C/T and IVS3 of the *IL4* gene, respectively, using the primers shown in Table S2. The restriction enzyme used was *Ava*II (Fermentas, Vilnius, Lithuania) for digesting amplicons of the -590 SNP. 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.

RNA extraction and cDNA synthesis

Total RNA from whole blood was isolated and purified using the Ribopure™ blood Kit (Ambion Inc., Austin, TX, U.S.A.) following the manufacturer's protocol. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas).

Determination of *IL4* and *GAPDH* mRNA expression by real-time polymerase chain reaction

The levels of *IL4* (target) and *GAPDH* (reference) transcripts were measured by real-time PCR using gene-specific primers (Eurofins, Bangalore, India) as shown in Table S2. Real-time PCR was performed in duplicates in 20 μL using LightCycler®480 SYBR Green I Master following the manufacturer's instructions and carried out in the Light Cycler®480 II Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). At the end of the amplification phase a melting curve analysis was carried out on the product formed. The fluorescent data collection was performed during the extension step. The value of Ct was determined by the first cycle number at which fluorescence was greater than the set threshold value. The data are shown as the ratio of Ct values of *IL4* and *GAPDH*.

Estimation of serum interleukin-4 levels by enzyme-linked immunosorbent assay

Serum levels of IL-4 in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA)

using the AviBion Human IL-4 ELISA kit (Ani Biotech Oy, Vantaa, Finland) as per the manufacturer's protocol.

Estimation of serum IgE levels by electrochemiluminescence immunoassay

Serum IgE levels in patients with vitiligo and controls were also monitored by electrochemiluminescence immunoassay (ECLIA) using the Human IgE ECLIA kit (Roche Diagnostics GmbH) as per the manufacturer's protocol.

Statistical analysis

The distribution of the genotypes and allele frequencies was compared using the χ^2 test with 3×2 and 2×2 contingency tables, respectively, using Prism 4 software (GraphPad Software Inc., San Diego, CA, U.S.A.). Vitiligo samples from North India and Gujarat were compared with their respective unaffected controls using χ^2 analysis and Fisher's exact test and strength of associations was estimated by odds ratio (OR) and 95% confidence interval (CI) using the Stata 9.2 statistical program (Statacorp, College Station, TX, U.S.A.). Haplotype analysis was carried out using SHEsis program.[18,19] The linkage disequilibrium (LD) coefficients $D' = D/D_{max}$ and r^2 values for the pair of the most common alleles at each site were also estimated using the Haploview program version 4.1.[20] Differences were considered to be statistically significant if the P-value was $\leq 0{\cdot}05$. Relative gene expression of IL4, serum IL-4 and IgE levels were plotted and analysed by nonparametric unpaired t-test using Prism 4 software. 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.[21]

Results

Association of *IL4* gene intron 3 VNTR with vitiligo

The three genotypes of IL4 IVS3 were classified as: R1 (183 bp–two 70 bp repeat allele), R2 (253 bp–three 70 bp repeat allele) and R1R2 (both 183 and 253 bp fragments) (Fig. S1a; see Supporting information).

IVS3 in the *IL4* gene was found to be associated with vitiligo susceptibility in the Gujarati population, as genotype and allele frequencies were significantly different ($P < 0{\cdot}0001$, $P < 0{\cdot}0001$, respectively) between the patients and controls. The allele frequencies of the R1 and R2 alleles differed significantly between patients with generalized vitiligo and controls from Gujarat ($P < 0{\cdot}0001$), with R2 being significantly increased in patients compared with controls; however, this was not the case in the North Indian population ($P = 0{\cdot}32$) (Table 1). Genotype R2R2 was significantly increased ($P = 0{\cdot}02$) and R1R1 was significantly reduced ($P < 0{\cdot}0001$) in patients with generalized vitiligo compared with controls (Table 1) in the Gujarat population. Only the R1R1 genotype was significantly reduced in the North Indian patients with generalized vitiligo compared with controls ($P = 0{\cdot}005$).

However, genotype and allele frequencies for IVS3 were not significantly different in patients with localized vitiligo compared with controls in either of the populations (Table 2). This study has 97% and 94% statistical power, respectively, for the Gujarat and North Indian populations for the effect size 0·1 to detect association of IVS3 in patients with vitiligo and the control population ($P < 0{\cdot}05$).

Association of -590 C/T promoter polymorphism in *IL4* gene with vitiligo

The genotyping of the -590 C/T SNP revealed a 195-bp undigested product corresponding to the T allele and 177- and 18-bp digested products corresponding to the C allele (Fig. S1b).

The -590 C/T SNP was found to be associated with vitiligo susceptibility in both the populations, as genotype and allele frequencies were significantly different ($P < 0{\cdot}0001$, $P < 0{\cdot}0001$, respectively) between patients and controls. The frequencies of the C and T alleles differed significantly between patients with generalized vitiligo and controls from Gujarat ($P < 0{\cdot}0001$) with the T allele being significantly increased in patients compared with controls; however, in North Indians, the C allele was significantly increased in patients with generalized vitiligo compared with controls ($P < 0{\cdot}0001$) (Table 1). Genotype TT was significantly increased ($P = 0{\cdot}005$) and CC was significantly reduced ($P = 0{\cdot}0002$) in patients with generalized vitiligo compared with controls (Table 1) in the Gujarat population; however, the CC genotype was significantly increased ($P < 0{\cdot}0001$) and the TT genotype was significantly reduced in the North Indian patients with vitiligo compared with controls ($P < 0{\cdot}0001$).

Interestingly, genotype and allele frequencies for the -590 C/T SNP also differed significantly between patients with localized vitiligo and controls in both the populations ($P = 0{\cdot}004$, $P = 0{\cdot}001$ for Gujarat and $P < 0{\cdot}0001$, $P < 0{\cdot}0001$ for North India) suggesting an association of -590 C/T polymorphism with localized vitiligo (Table 2). This study has 97% and 93% statistical power, respectively, for Gujarat and North Indian populations for the effect size 0·1 to detect an association of -590C/T SNP at $P < 0{\cdot}05$ in patients and the control population.

Linkage disequilibrium and haplotype analyses

As -590C/T and IVS3 are on the same gene it is important to know which of the alleles at the two regions are present together on the same chromosome, i.e. which alleles are in linkage disequilibrium (LD) and make haplotypes. The LD analysis revealed that the two polymorphisms investigated in the IL4 gene were in low LD with $D' = 0{\cdot}072$ and 0·275 in Gujarati and North Indian patients with generalized vitiligo, and $D' = 0{\cdot}159$ and 0·277 in patients with localized vitiligo from Gujarat and North India, respectively.

The estimated frequencies of the haplotypes differed significantly between patients with generalized vitiligo and controls

Table 1 Association study for *IL4* gene intron 3 VNTR (IVS3) and -590 C/T single nucleotide polymorphism (SNP) in patients with generalized vitiligo and controls from Gujarat and North India

Population	SNP/genotype	Patients		Controls		P-value	Odds ratio (95% CI)	P-value (global)
		n	Frequency	n	Frequency			
Gujarat	Intron 3 VNTR (IVS3)	395		744				
	Genotype							
	R1R1	23	0·06	106	0·14	0·0001	0·3 (0·2–0·6)	< 0·0001
	R1R2	107	0·27	188	0·25	0·50	1·0 (0·8–1·4)	
	R2R2	265	0·67	450	0·61	0·02	1·3 (1·0–1·7)	
	Allele							
	R1	153	0·19	400	0·27	0·0001	0·6 (0·5–0·8)	< 0·0001
	R2	637	0·81	1088	0·73	0·0001	1·5 (1·2–1·9)	
North India	(IVS3)	464		397				
	Genotype							
	R1R1	13	0·03	27	0·07	0·005	0·3 (0·1–0·8)	0·013
	R1R2	140	0·30	103	0·26	0·16	1·2 (0·9–1·6)	
	R2R2	311	0·67	267	0·67	0·94	0·9 (0·7–1·3)	
	Allele							
	R1	166	0·18	157	0·20	0·31	0·8 (0·6–1·1)	0·323
	R2	762	0·82	637	0·80	0·31	1·1 (0·8–1·4)	
Gujarat	-590 C/T (rs2243250)	395		646				
	Genotype							
	CC	169	0·43	353	0·55	0·0002	0·6 (0·4–0·8)	0·001
	CT	99	0·25	136	0·21	0·13	1·2 (0·9–1·7)	
	TT	127	0·32	157	0·24	0·005	1·4 (1·1–1·9)	
	Allele							
	C	437	0·55	842	0·65	$7·30 \times 10^{6}$	0·6 (0·5–0·7)	< 0·0001
	T	353	0·45	450	0·35	$7·30 \times 10^{6}$	1·5 (1·2–1·8)	
North India	-590 C/T (rs2243250)	464		389				
	Genotype							
	CC	286	0·62	115	0·29	$8·90 \times 10^{21}$	3·8 (2·8–5·1)	< 0·0001
	CT	131	0·28	100	0·26	0·40	1·1 (0·8–1·5)	
	TT	47	0·10	174	0·45	$1·50 \times 10^{30}$	0·13 (0–0·2)	
	Allele							
	C	703	0·75	330	0·42	$9·80 \times 10^{45}$	4·2 (3·4–5·2)	< 0·0001
	T	225	0·25	448	0·58	$9·80 \times 10^{45}$	0·2 (0·1–0·2)	

CI, confidence interval.

in Gujarati and North Indian populations (global $P < 10^{-8}$, $P < 10^{-16}$, respectively) (Table 3). Also, patients with localized vitiligo exhibited significantly different frequencies of haplotypes compared with controls in Gujarat and North Indian populations (global $P < 10^{-5}$, $P < 10^{-30}$, respectively (Table 4).

The results showed that haplotype TR2 was significantly increased ($P < 10^{-9}$) in patients with generalized vitiligo from Gujarat and haplotype CR1 was significantly reduced ($P = 0·001$) compared with controls (Table 3). On the other hand, haplotype CR2 was significantly increased ($P < 10^{-15}$) in patients with generalized vitiligo from North India and TR2 was significantly reduced ($P < 10^{-16}$) compared with the ethnically matched controls. Haplotype analysis for patients with localized vitiligo and controls showed that the TR1 haplotype was increased significantly ($P = 0·031$) in the patients from Gujarat and the CR1 haplotype was reduced significantly compared with controls ($P < 10^{-5}$). The CR2 haplotype was significantly increased ($P < 10^{-15}$) and TR2 was significantly reduced ($P < 10^{-15}$) in patients with localized vitiligo from North India (Table 4). The differences observed in the Gujaratis and North Indians could be due to different ethnicity as they form two independent endogamous groups which do not intermarry.

Age of onset of vitiligo and *IL4* haplotypes in patients with vitiligo

In addition, age of onset of the disease and IL4 haplotypes were also correlated in patients with vitiligo from Gujarat and North India. Interestingly, Gujarati patients with vitiligo with the TTR2R2 haplotype had an early onset of vitiligo (mean age ± SD, 9·5 ± 0·8660 years) compared with the CCR1R2 and TTR1R1 haplotypes ($P = 0·027$; $P = 0·005$, respectively) (Fig. 1a). Also, patients with the TTR1R2 haplotype had early onset of vitiligo (mean age ± SD, 11·29 ± 1·742 years) com-

Table 2 Association study for *IL4* gene intron 3 VNTR (IVS3) and -590 C/T single nucleotide polymorphism (SNP) in patients with localized vitiligo and controls from Gujarat and North India

Population	SNP/genotype	Patients n	Patients Frequency	Controls n	Controls Frequency	P-value	Odds ratio (95% CI)	P-value (global)
Gujarat	Intron 3 VNTR (IVS3)	110		744				
	Genotype							
	R1R1	15	0·13	106	0·15	0·80	0·9 (0·4–1·7)	0·807
	R1R2	25	0·23	188	0·25	0·50	0·8 (0·5–1·4)	
	R2R2	70	0·64	450	0·60	0·50	1·1 (0·7–1·7)	
	Allele							
	R1	55	0·25	400	0·27	0·61	0·9 (0·6–1·2)	0·624
	R2	165	0·75	1088	0·73	0·61	1·1 (0·7–1·5)	
North India	IVS3	132		397				
	Genotype							
	R1R1	7	0·05	27	0·07	0·50	0·7 (0·2–1·8)	0·734
	R1R2	38	0·29	103	0·26	0·50	1·1 (0·7–1·8)	
	R2R2	87	0·66	267	0·67	0·70	0·9 (0·6–1·4)	
	Allele							
	R1	52	0·20	157	0·20	0·90	0·9 (0·6–1·4)	0·930
	R2	212	0·80	637	0·80	0·90	1·0 (0·7–1·4)	
Gujarat	-590 C/T	110		646				
	Genotype							
	CC	43	0·39	353	0·55	0·002	0·5 (0·3–0·8)	0·004
	CT	37	0·34	136	0·21	0·003	1·9 (1·1–3·0)	
	TT	30	0·27	157	0·24	0·500	1·1 (0·7–1·8)	
	Allele							
	C	123	0·56	842	0·65	0·008	0·6 (0·5–0·9)	0·001
	T	97	0·44	450	0·35	0·008	1·4 (1·0–1·9)	
North India	-590 C/T (rs2243250)	132		389				
	Genotype							
	CC	82	0·62	115	0·29	$2·60 \times 10^{11}$	3·9 (2·5–6·0)	< 0·0001
	CT	34	0·25	100	0·26	0·9	1·1 (0·6–1·6)	
	TT	16	0·13	174	0·45	$1·70 \times 10^{11}$	0·17 (0·09–0·3)	
	Allele							
	C	198	0·75	330	0·42	$5·60 \times 10^{20}$	4 (2·9–5·6)	< 0·0001
	T	66	0·25	448	0·58	$5·60 \times 10^{20}$	0·24 (0·1–0·3)	

CI, confidence interval.

Table 3 Haplotypes of *IL4* -590 C/T and intron 3 VNTR showing significant differences between patients with generalized vitiligo and controls from Gujarat and North India

Population	Haplotype (-590 C/T and intron 3 VNTR)	Patients Frequency (%)	Controls Frequency (%)	P-value	P-value (global)	Odds ratio (95% CI)
Gujarat		2n = 598	2n = 1246			
	CR1	9·00	14·33	0·001	$< 10^{-8}$	0·590 (0·428–0·815)
	CR2	49·70	5·25	0·026		0·801 (0·658–0·973)
	TR1	7·10	8·70	0·229		0·798 (0·551–1·154)
	TR2	34·20	21·72	$< 10^{-9}$		1·877 (1·512–2·329)
North India		2n = 898	2n = 650			
	CR1	7·69	7·55	0·914	$< 10^{-16}$	1·021 (0·698–1·494)
	CR2	68·59	33·68	$< 10^{-15}$		4·298 (3·466–5·331)
	TR1	9·79	11·84	0·197		0·808 (0·584–1·118)
	TR2	13·93	46·93	$< 10^{-16}$		0·183 (0·143–0·234)

CI, confidence interval.

Table 4 Haplotypes of IL4 -590 C/T and intron 3 VNTR showing significant differences between patients with localized vitiligo and controls from Gujarat and North India

Population	Haplotype (-590 C/T and intron 3 VNTR)	Patients Frequency (%)	Controls Frequency (%)	P-value	P-value (global)	Odds ratio (95% CI)
Gujarat		2n = 184	2n = 1246			
	CR1	2·67	14·33	$< 10^{-5}$	$< 1\ 0^{-5}$	0·164 (0·066–0·408)
	CR2	60·37	55·25	0·191		1·234 (0·900–1·692)
	TR1	13·63	8·70	0·031		1·657 (1·041–2·637)
	TR2	23·33	21·72	0·623		1·096 (0·759–1·583)
North India		2n = 264	2n = 650			
	CR1	4·12	7·55	0·058	$< 10^{-30}$	1·021 (0·698–1·494)
	CR2	71·64	33·68	$< 10^{-15}$		4·298 (3·466–5·331)
	TR1	14·82	11·84	0·220		0·808 (0·584–1·118)
	TR2	9·42	46·93	$< 1\ 0^{-15}$		0·183 (0·143–0·234)

CI, confidence interval.

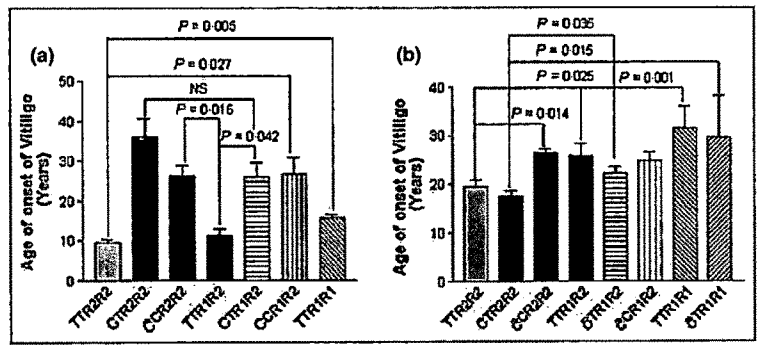

Fig 1. Comparison of age of onset of vitiligo with IL4 haplotypes in patients with vitiligo from (a) Gujarat and (b) North India.

pared with those with CCR2R2 and CTR1R2 (P = 0·016; P = 0·042, respectively) (Fig. 1a).

Interestingly, North Indian patients with vitiligo with the TTR2R2 haplotype also had an early onset of the disease (mean age ± SD, 19·56 ± 1·283 years) compared with those with CCR2R2, TTR1R2 and TTR1R1 (P = 0·014; P = 0·025; P = 0·0013, respectively). Moreover, patients with the CTR2R2 haplotype also had early onset of vitiligo (mean age ± SD, 17·54 ± 1·194 years) compared those with CTR1R1 and CTR1R2 (P = 0·015; P = 0·035, respectively) (Fig. 1b).

Relative gene expression of *IL4* in patients with generalized vitiligo and controls from Gujarat

Gene expression studies revealed higher expression of IL4 in 84 patients with generalized vitiligo compared with 93 controls (P = 0·001) (Fig. 2a). Further, the expression levels of IL4 were analysed with respect to haplotypes generated from the two investigated polymorphisms of IL4 (Fig. 2b). IL4 mRNA expression differed significantly with respect to haplotypes generated at -590 and IVS3 loci, i.e. CCR2R2, CTR2R2

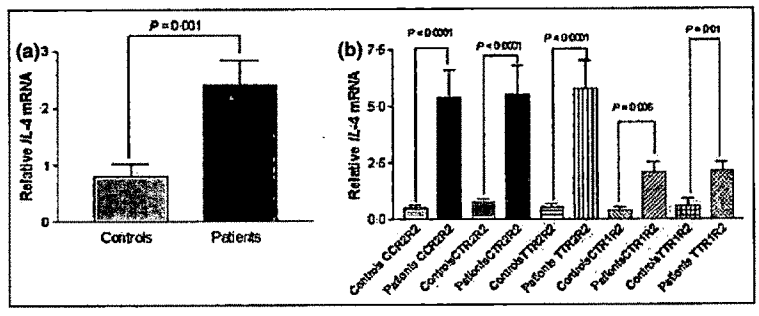

Fig 2. Relative gene expression and genotype–phenotype correlation for IL4 transcript in controls and patients with generalized vitiligo. (a) Relative gene expression of IL4 in 84 patients with generalized vitiligo and 93 controls as suggested by ratio of target (IL4 transcripts)/reference (GAPDH transcripts). (b) Relative mRNA expression of IL4 with respect to -590 C/T and intron 3 VNTR haplotypes in 84 patients with generalized vitiligo and 93 controls as suggested by ratio of target (IL4 transcripts)/reference (GAPDH transcripts).

and TTR2R2 ($P < 0{\cdot}0001$; $P < 0{\cdot}0001$; $P < 0{\cdot}0001$, respectively) in patients with vitiligo compared with controls. Also, the CTR1R2 and TTR1R2 genotypes revealed significantly higher mRNA expression ($P = 0{\cdot}006$; $P = 0{\cdot}01$, respectively) in patients compared with controls.

Estimation of serum interleukin-4 levels and its correlation with investigated polymorphisms in patients with generalized vitiligo and controls from Gujarat

Serum IL-4 levels in 86 patients with generalized vitiligo and 95 controls were estimated by ELISA. IL-4 levels were found to be significantly different in patients with vitiligo compared with controls ($P < 0{\cdot}0001$) (Fig. 3a). In addition, serum IL-4 levels were analysed with respect to haplotypes generated at the -590 and IVS3 loci. IL-4 levels were significantly higher in patients for the CCR2R2, CTR2R2, CTR1R2 and TTR2R2 genotypes ($P = 0{\cdot}002$; $P = 0{\cdot}013$; $P = 0{\cdot}044$ and $P = 0{\cdot}002$, respectively) (Fig. 3b).

Estimation of serum IgE levels and its correlation with investigated polymorphisms in patients with generalized vitiligo and controls from Gujarat

We also measured serum IgE levels in 82 patients with generalized vitiligo and 90 controls from Gujarat. Patients with vitiligo showed increased IgE levels compared with controls ($P = 0{\cdot}019$) (Fig. 4a). Moreover, when serum IL-4 levels were compared with respect to haplotypes generated at -590 and IVS3 loci, the IgE levels were significantly increased for TTR2R2, CTR2R2, CCR2R2, TTR1R2 and CTR1R2 haplotypes ($P = 0{\cdot}003$, $P = 0{\cdot}014$, $P = 0{\cdot}033$, $P = 0{\cdot}006$ and $P = 0{\cdot}026$, respectively) in patients compared with controls (Fig. 4b). However, no significant difference was observed for serum IgE levels between patients and controls for CCR1R2 and TTR1R1 haplotypes ($P = 0{\cdot}579$, $P = 0{\cdot}587$, respectively) (Fig. 4b).

Discussion

Although the aetiology of vitiligo remains obscure, autoimmunity has been suggested to play a major role in its pathogenesis.[5] Our previous study suggested that 22% of patients with vitiligo from Gujarat exhibit a positive family history and 14% patients have at least one-first-degree relative affected.[22] The destruction of melanocytes due to an autoimmune response in vitiligo can be either through cellular and/or humoral immune response.[4,5] We have also shown that 66% of patients with vitiligo possess antimelanocyte antibodies in their circulation compared with a control population.[23] Recently, we showed a positive association of *HLA-A**33:01, *HLA-B**44:03 and *HLA-DRB1**07:01 with vitiligo in patients from North India and Gujarat suggesting an autoimmune link of vitiligo in these cohorts.[24] The genotype–phenotype correlation of *CTLA4* gene

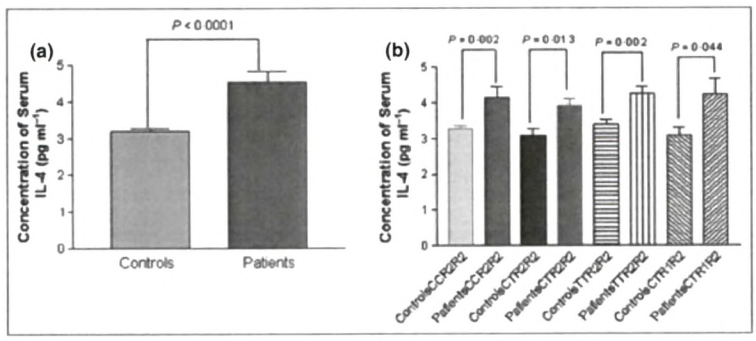

Fig 3. Correlation of serum interleukin (IL)-4 levels with investigated polymorphisms in controls and patients with generalized vitiligo. (a) Comparison of serum IL-4 levels (pg mL^{-1}) in 86 patients with generalized vitiligo and 95 controls as determined by enzyme-linked immunosorbent assay. (b) Comparison of serum IL-4 levels (pg mL^{-1}) in 86 patients with generalized vitiligo and 95 controls with respect to haplotypes based on the investigated polymorphisms.

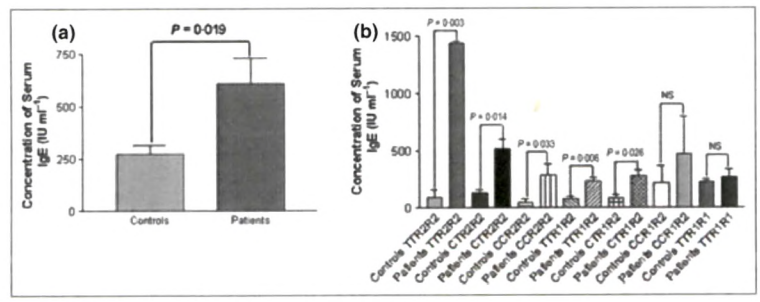

Fig 4. Correlation of serum IgE levels with investigated polymorphisms in controls and patients with generalized vitiligo. (a) Comparison of serum IgE levels (IU mL^{-1}) in 82 patients with generalized vitiligo and 90 controls as determined by electrochemiluminescence immunoassay. (b) Comparison of serum IgE levels (IU mL^{-1}) in 82 patients with generalized vitiligo and 90 controls with respect to haplotypes based on the investigated polymorphisms.

polymorphisms also supports the autoimmune pathogenesis of vitiligo in the Gujarat population whereas our earlier studies on *MBL2*, *ACE* and PTPN22 polymorphisms did not show significant association.[25–28]

In this study, we investigated two well-documented IL4 polymorphisms, -590 C/T SNP and IVS3, in patients with vitiligo from Gujarat and North India. The -590 T allele is associated with increased promoter activity, a higher proportion of IL-4-producing Th cells,[17] and elevated serum IgE level.[15] One study by Pehlivan *et al.*[29] in 2009 reported its nonassociation with vitiligo in a Turkish population. Interestingly, ours is the first report suggesting a strong association of -590 C/T polymorphism with vitiligo susceptibility. We found that the susceptible (TT) genotype frequency is higher in patients from Gujarat with generalized and with localized vitiligo compared with controls, suggesting the profound effect of -590 C/T polymorphism in both the types of vitiligo. This particular genotype confers higher expression of IL4, as the T allele is known to increase transcriptional activity[15] and thereby also upregulates IL-4 protein expression. However, the CC genotype frequency was higher in patients with vitiligo from North India, suggesting a difference in genetic predisposition due to ethnicity differences between the two populations. Also, higher frequency of the low producing genotype CC in North Indian patients may implicate the role of Th1 type of autoimmune responses in these patients compared with higher Th2 responses in Gujarat. It is pertinent to mention here that the prevalence of vitiligo in Gujarat is higher than in North India and it is possible that different autoimmune aetiologies may be playing a role in the manifestation of the disease in the two ethnic groups in India. The IL4 -590 C/T promoter polymorphism is associated with autoimmune disorders such as allergic asthma and systemic lupus erythematosus (SLE).[30,31] The IL4 -590 TT genotype was significantly more frequent in patients with rheumatoid arthritis (RA) than in controls.[32] Nevertheless there are contradictory reports which suggest that this polymorphism is not associated with autoimmune disorders such as autoimmune thyroid disease[33] and Graves disease.[34]

The present study also addressed IVS3 in patients with vitiligo and controls and found a significant association with vitiligo susceptibility in the Gujarati population. It is suggested that a distinct number of VNTR copies might affect the transcriptional activity of the IL4 gene.[35] The three repeat allele (R2) is known to be high producer of IL-4,[17] which was found to be higher in patients with generalized vitiligo from Gujarat; however the allele has no association with patients with localized vitiligo from Gujarat suggesting that it may have a crucial role in generalized vitiligo. Previously, this polymorphism was reported to be associated with RA and immune thrombocytopenic purpura and SLE.[35,36] One North Indian study also suggested an association of IVS3 with susceptibility to type-2 diabetes.[37] In contrast, this polymorphism was not found to be associated with autoimmune thyroid disease.[33] This is the first report investigating the role of IVS3 and suggesting its positive association with vitiligo.

Our relative gene expression studies and ELISA results showed a significant increase in IL4 transcript and protein levels in Gujarati patients with vitiligo suggesting its crucial role in vitiligo pathogenesis. This is the first report where IL4 expression and protein levels were studied in patients with vitiligo. Further, considering the IL4 -590 C/T promoter polymorphism, we found that patients with the CT and TT genotypes revealed higher IL4 mRNA expression. Also, the CT and TT genotypes showed significant differences in serum IL-4 levels in Gujarati patients with vitiligo compared with controls. In a recent study by Kim *et al.*,[38] the -590 C/T SNP was shown to be associated with higher IL4 mRNA expression in patients with asthma. Our results document the same evidence in patients with vitiligo. We found that patients with vitiligo harbouring the CCR2R2, CTR2R2 and TTR2R2 haplotypes showed significantly increased mRNA and protein levels compared with controls, revealing the profound effect of the R2R2 genotype on IL-4 expression.

In addition, higher serum IgE levels in Gujarati patients with vitiligo suggest that this increase could be due to increased IL-4 levels. Further, the -590 TT and IVS3 R2R2 genotypes were found to be associated with increased serum IgE levels. Our results are in accordance with those of Guia and Ramos, demonstrating the association of -590 TT genotype with elevated serum IgE levels in individuals with atopic allergy.[39] Our results showed that patients with vitiligo with the CCR2R2, CTR2R2 and TTR2R2 haplotypes showed significantly increased serum IgE levels compared with controls, revealing the intense effect of the R2R2 genotype.

Previous studies also showed an increase in total IgE count in 59 patients with vitiligo and these patients had a significantly higher incidence of vitiligo in their families, an earlier onset and a rapid worsening of the disease.[40,41] In particular, analysis of age of onset of the disease in patients suggests that TTR2R2, TTR1R2 and CTR2R2 had a significant effect in the early onset of the disease and further can also be supported by the increased serum IL-4 and IgE levels in patients with these haplotypes. Thus, our findings strongly support the fact that the T and R2 alleles are associated with increased promoter activity,[15] a higher proportion of IL-4-producing Th cells[17] and elevated serum IgE levels.[15] Considering IL4 IVS3, the R2 allele is known to be a higher producer of IL-4.[17]

In summary, our present study on the well-documented IL4 gene polymorphisms reveals the crucial role of IL-4 in vitiligo susceptibility of the Gujarati population; however, only the -590 C/T polymorphism showed significant association with vitiligo in the North Indian population. The differences in the results in the two populations studied seem very interesting and suggest different pathways may be involved in achieving the same goal, i.e. vitiligo. It is well known that IL-4 has been shown to stimulate B-cell proliferation, to regulate immunoglobulin class switching (IgG1 and IgE), and to promote T-cell development,[10] and therefore plays an important role in precipitating autoimmune responses. Considering the fact that IL-4 is one of the most important Th2 cytokines, and increased IL-4 levels induce a balance shift from Th1 to Th2

cells, determining the molecular profile of a particular person becomes crucial in understanding the immune reaction mechanisms. In cases of atopic dermatitis where hyper-produced Th1 and Th2 cytokines are involved, the initiation phase of the disease involved increased IL-4 production by Th2 cells.[42] Previously, an *in vivo* study showed that IL-4 contributed to increased levels of IgE and IgG1 in mice treated with mercuric chloride, which led to a systemic autoimmune disease, emphasizing the role of IL-4 in humoral immune response-mediated pathogenesis.[43]

We therefore hypothesize that IL-4 plays a crucial role in aggravating immune responses in Gujarati patients with vitiligo, and its ability to stimulate B-cell proliferation may be involved in precipitating the humoral immune responses (Th2) in these patients. Also, for the first time, we document the role of the IL4 -590 C/T promoter polymorphism as well as IVS3 in increased expression of IL4 transcript in correlation to higher IL-4 and serum IgE levels in patients with vitiligo and thereby conferring susceptibility towards vitiligo in the Gujarati population.

What's already known about this topic?

- Interleukin (IL)-4 has been shown to stimulate B-cell proliferation, to regulate immunoglobulin class switching (IgG1 and IgE) and to promote T-cell development, and therefore may play an important role in autoimmunity.

What does this study add?

- The present study suggests that intron 3 VNTR and -590 C/T single nucleotide polymorphism of IL4 may be genetic risk factors for vitiligo susceptibility in the Gujarati population and be responsible for the altered levels of IL-4 and IgE in patients with vitiligo, thereby indicating the crucial role of IL-4 in autoimmune pathogenesis of vitiligo.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis of *IL4* -590 C/T polymorphism on 10% polyacrylamide gel electrophoresis. Lanes 1, 3 and 5, heterozygous (CT) genotypes; lane 2, homozygous (CC) genotype; lane 4, homozygous (TT) genotype; lane M, 100 bp DNA ladder. (b) PCR analysis of *IL4* intron 3 VNTR polymorphism on 2% agarose gel electrophoresis. Lanes 1, 5 and 8, homozygous (R2R2) genotypes; lanes 2 and 6, heterozygous (R1R2) genotypes; lanes 3, 4 and 7, homozygous (R1R1) genotypes; lane M, 50 bp DNA ladder.

Table S1. Demographic characteristics of patients with vitiligo and unaffected controls from Gujarat and North India.

Table S2. Primers used for genotyping of *IL4* intron 3 VNTR (IVS3) and -590 C/T single nucleotide polymorphism and *IL4* gene expression analysis.

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