BJD
British Journal of Dermatology

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

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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.

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**Accepted for publication**
2 April 2012

**Funding sources**
*Department of Biotechnology grant to R.B. (BT/PR9024/MED/12/332/2007) New Delhi, India, University Grants Commission grant to R.B. [F. No. 36-158/2008(SR)], New Delhi, India and Gujarat State Biotechnology Mission grant to R.B. (GSBTM/MD/PROJECTS/SSA/453/2010-2011), Gujarat, India.*

**Conflicts of interest**
*None declared.*

*M.I. and N.C.L. contributed equally to this work.*

*DOI 10.1111/j.1365-2133.2012.11000.x*

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 $\chi^2$ test with $3 \times 2$ and $2 \times 2$ 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 $\chi^2$ 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

| | | Patients | | Controls | | | | |
|---|---|---|---|---|---|---|---|---|
| Population | SNP/genotype | n | Frequency | n | Frequency | P-value | Odds ratio (95% CI) | P-value (global) |
| 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 | $7{\cdot}30 \times 10^{6}$ | 0·6 (0·5–0·7) | < 0·0001 |
| | T | 353 | 0·45 | 450 | 0·35 | $7{\cdot}30 \times 10^{6}$ | 1·5 (1·2–1·8) | |
| North India | -590 C/T (rs2243250) | 464 | | 389 | | | | |
| | Genotype | | | | | | | |
| | CC | 286 | 0·62 | 115 | 0·29 | $8{\cdot}90 \times 10^{21}$ | 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 | $1{\cdot}50 \times 10^{30}$ | 0·13 (0–0·2) | |
| | Allele | | | | | | | |
| | C | 703 | 0·75 | 330 | 0·42 | $9{\cdot}80 \times 10^{45}$ | 4·2 (3·4–5·2) | < 0·0001 |
| | T | 225 | 0·25 | 448 | 0·58 | $9{\cdot}80 \times 10^{45}$ | 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 ($P = 0{\cdot}001$) 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 ($P = 0{\cdot}031$) 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 ($P = 0{\cdot}027$; $P = 0{\cdot}005$, 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

| | | Patients | | Controls | | | | |
|---|---|---|---|---|---|---|---|---|
| Population | SNP/genotype | n | Frequency | n | 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 | $2{\cdot}60 \times 10^{11}$ | 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 | $1{\cdot}70 \times 10^{11}$ | 0·17 (0·09–0·3) | |
| | Allele | | | | | | | |
| | C | 198 | 0·75 | 330 | 0·42 | $5{\cdot}60 \times 10^{20}$ | 4 (2·9–5·6) | < 0·0001 |
| | T | 66 | 0·25 | 448 | 0·58 | $5{\cdot}60 \times 10^{20}$ | 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.

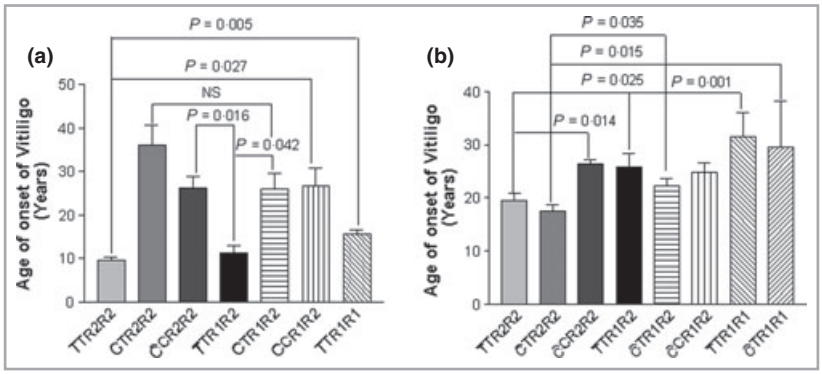


**Fig 1.** Comparison of age of onset of vitiligo with IL4 haplotypes in patients with vitiligo from (a) Gujarat and (b) North India.

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

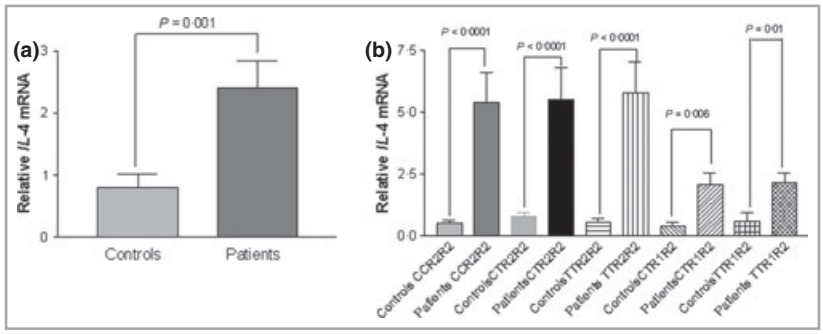


**Fig 2.** Relative gene expression and genotype–phenotype correlation for IL4 transcript in controls and patients with generalized vitiligo. (a) Relative gene expression of IL4 in 84 patients with generalized vitiligo and 93 controls as suggested by ratio of target (IL4 transcripts)/reference (*GAPDH* 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

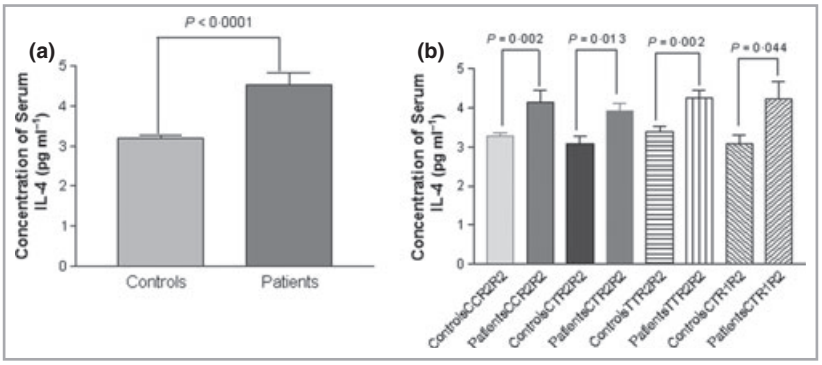


**Fig 3.** Correlation of serum interleukin (IL)-4 levels with investigated polymorphisms in controls and patients with generalized vitiligo. (a) Comparison of serum IL-4 levels (pg $mL^{-1}$) in 86 patients with generalized vitiligo and 95 controls as determined by enzyme-linked immunosorbent assay.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

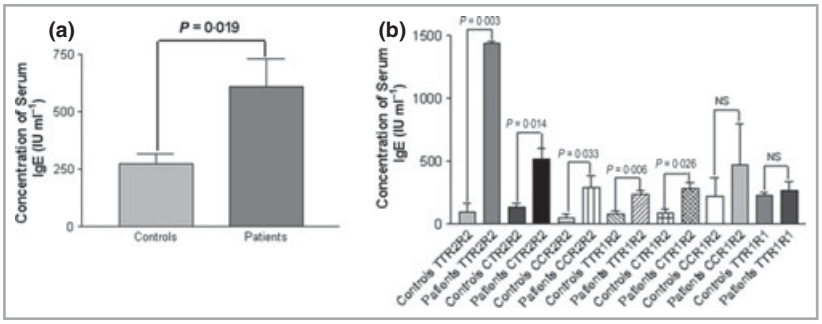


**Fig 4.** Correlation of serum IgE levels with investigated polymorphisms in controls and patients with generalized vitiligo. (a) Comparison of serum IgE levels (IU $mL^{-1}$) in 82 patients with generalized vitiligo and 90 controls as determined by electrochemiluminescence immunoassay. (b) Comparison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

## Acknowledgments

We thank all the patients with vitiligo and the control subjects for their participation in this study. N.C.L. thanks the Council of Scientific and Industrial Research (New Delhi) for awarding a SRF. We also thank Dr Yongyong Shi for helping us in linkage disequilibrium and haplotype analysis. We are thankful to Toprani Advanced Lab Systems, Vadodara, Gujarat, India for assisting us in measuring serum IgE levels.

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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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