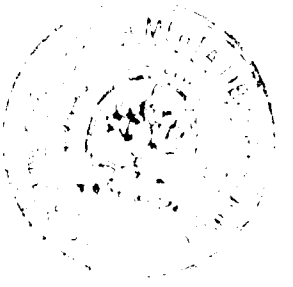

SYNOPSIS

INTRODUCTION

Genes involved in common complex diseases such as autoimmune disorders that affect approximately 5% of the population remain obscure. Vitiligo is an acquired hypomelanotic disorder characterized by circumscribed depigmented macules in the skin resulting from loss of functional melanocytes from the 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 (Taieb et al., 2007) and in India the incidence of vitiligo is found to be 0.5-2.5% (Handa and Kaur, 1999). The etiology 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 vitiligo patients compared to unaffected individuals (Kemp et al., 2001).

Various factors such as oxidative stress, genetic factors, neural factors, toxic metabolites and lack of melanocyte growth factors might contribute for precipitating the disease in susceptible people (Njoo and Westerhof, 2001). The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Nordlund, 1997). It has been found that about 20% of vitiligo patients have at least one first-degree relative affected (Nath et al., 1994). Our study also showed that 22% of Gujarat vitiliginous patients exhibit positive family history and 14% patients have at least one first-degree relative affected (Shajil et al., 2006). Vitiligo 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 role in regulating immunity have been found to be associated with susceptibility to vitiligo including allelic variants in the cytotoxic T -lymphocyte antigen-4 gene (*CTLA4*) (Blomhoff et al., 2005), the autoimmune susceptibility loci (*AIS*1, *AIS*2, *AIS*3 and *SLEV*1) (Alkhateeb et al., 2001; Fain et al., 2003; Spritz et al., 2004), the autoimmune regulator (*AIRE*) gene (Nagamine et al., 1997), NACHT leucine-rich-repeat protein 1 (*NALP*1- maps at *SLEV*1 sucsceptibility locus) (Jin et al., 2007), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) (Laberge et al., 2008) and certain human leukocyte antigen specificities of the major histocompatibility complex (Le Poole et al., 2008; Fain et al., 2006).

Most of the population studies on vitiligo are done in western countries; however clinical studies on Indian population are scarce. Since the environmental factors could account for the origin of autoimmunity in the susceptible patients, clinical and genetic studies on vitiligo in Indian patients are required. In order to explore the genetic susceptibility, identification and systematic study of genes involved in regulation of autoimmunity is essential. Attempts to identify genes involved in vitiligo susceptibility have involved gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses. The aim of present study was to assess role of autoimmunity in pathogenesis of vitiligo through candidate gene approach.

Objectives of the present study:

1. Estimation of antimelanocyte antibody levels in Gujarat vitiligo patients compared to controls.
2. Genetic association of Protein Tyrosine Phosphatase Non receptor 22 *(PTPN22)* and Angiotensin Converting Enzyme *(ACE)* genes polymorphisms with vitiligo susceptibility.
3. Role of Mannan Binding Lectin-2 (*MBL2*) gene structural and promoter polymorphisms in vitiligo susceptibility.
4. Role of Interferon-γ (*IFN-γ*) gene intron 1 polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility.
5. Role of Cytotoxic T-Lymphocyte Associated antigen-4 (*CTLA-4*) gene polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility.
6. Role of Melanocyte Proliferating Gene 1 (*MYG1*) promoter and structural polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility.
7. Role of NACHT Leucine-rich Repeat Protein 1 (*NALP1*) gene promoter and structural polymorphisms in vitiligo pathogenesis.
8. Determination of $CD4^+/CD8^+$ ratio and estimation of $CD4^+CD25^+$FoxP3 T-regulatory cells (Tregs) in vitiligo patients and controls.

1. Estimation of antimelanocyte antibody levels in Gujarat vitiligo patients compared to controls:

We have analyzed plasma samples of 300 vitiligo patients and 400 controls for the presence of antimelanocyte antibodies by ELISA. A significant increase in the antimelanocyte antibody levels was seen in vitiligo patients compared to controls ($p=0.001$). Seventy five percent of vitiligo patients had antimelanocyte antibodies in their circulation suggesting that autoimmunity may play an important role in vitiligo pathogenesis.

2. Genetic association of Protein Tyrosine Phosphatase Non receptor 22 *(PTPN22)* and Angiotensin Converting Enzyme *(ACE)* genes polymorphisms with vitiligo susceptibility:

A) Genotyping of PTPN22 1858C/T (rs2476601) polymorphism in vitiligo patients and controls of Gujarat:

Protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene is known to play an important role in negative T cell regulation. *PTPN22* C/T single nucleotide polymorphism at 1858 (rs2476601) position was reported to be associated with increased risk of autoimmune diseases including generalized vitiligo (Canton et al., 2005). A total of 126 vitiligo patients and 140 age matched healthy controls were analyzed for the 1858 C/T polymorphism. The genotype and allele frequencies of this C/T SNP did not differ significantly between control and patient populations ($p= 0.198$; $p=0.560$ respectively) suggesting that there is no association of the C/T (*XcmI*) *PTPN22* marker with vitiligo.

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 (Ban et al., 2005) Chinese (Begovich et al., 2004) and South Asian Indians (Ray et al., 2006). Thus, our results suggest that the 1858T allele may not be a predisposing factor in Gujarat population that contributes in development of vitiligo.

B) Genotyping of ACE I/D polymorphism (NCBI: AF118569) in vitiligo patients and controls of Gujarat:

Angiotensin converting enzyme (*ACE*) has been found to be associated with several autoimmune disorders (Scholzen et al., 2003). 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).

We investigated the distribution of *ACE* I/D genotypes in 125 vitiligo patients and 156 ethnically matched healthy controls of Gujarat population to find the relationship between *ACE* I/D polymorphism and vitiligo. 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), suggesting that there is no association of the *ACE* I/D polymorphism with vitiligo. The allele frequencies of *ACE* I/D polymorphism did not differ significantly between the controls and patient population (p=0.252).

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) suggesting that the well documented I/D polymorphism in intron 16 of angiotensin converting enzyme in Gujarat population may not be associated with vitiligo susceptibility.

3. Genetic association of Mannan binding lectin-2 (*MBL2*) gene structural and promoter polymorphisms with vitiligo susceptibility:

Mannan-binding lectin (MBL) [mannose binding lectin (MBL)] is a liver-derived calcium dependent serum protein, which plays an important role in innate immune defense. Possible role of MBL in vitiligo pathogenesis could be due to defective clearance of apoptotic cells which may substantially contribute to the triggering of

autoimmune responses (Werth et al., 2002). The functional *MBL2* gene is located on chromosome 10 (q11.2-q21) and comprises of four exons. Three functional single-nucleotide polymorphisms (SNPs) in exon 1 of *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) (Lipscombe et al., 1992). 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 to 5 μg/ml) (Garred et al., 2003).
We investigated the association of *MBL2*-deficient genotypes of both promoter and exon 1 with vitiligo susceptibility in Gujarat population. A total of 92 vitiligo patients and 94 unaffected age matched controls of Gujarat population participated in this study, out of which 62 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 -221 promoter polymorphism, 92 patients and 93 control subjects were used. The genotyping of *MBL2* structural and promoter polymorphisms were carried out by heteroduplex analysis.

A) Genotyping of MBL2 codon 52 (rs5030737), codon 54 (rs1800450) and codon 57 (rs1800451) polymorphisms in vitiligo patients and controls of Gujarat:

Codon 52, codon 54 and codon 57 polymorphisms of MBL2 exon 1 were not found to be associated with vitiligo patients (p=0.019 for codon 52, p=0.373 for codon 54, p=0.855 for codon 57). Also, there was no significant difference in allele frequencies of codon 52, codon 54 and codon 57 between patients and controls (p=0.020 for D allele, p=0.378 for B allele and p=0.858 for C allele).
The association study for exon 1 polymorphisms was also done by A/O genotyping where all the three codon polymorphisms were placed in one group designated as 'O' allele and wild type as 'A' allele. There was no significant association found between the A/O genotype and vitiligo (p=0.999). Furthermore, 'O' allele was also not significantly associated with any of the population (p=0.968).

B) Genotyping of MBL2 -221 promoter polymorphism (rs7096206) in vitiligo patients and controls of Gujarat:

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. There was no significant association found between -221 promoter polymorphism and the risk of vitiligo (p=0.889). Also, the allele frequencies of patients and controls for -221 (X/Y) promoter polymorphism did not show significant difference (p=0.765).

Haplotype analysis:

The promoter and structural genotypes of patients and controls were combined to construct haplotypes and to find whether there was any haplotype associated with vitiligo susceptibility. Interestingly, no haplotype was found to be associated with vitiligo when compared with chi-square test (p=0.962). 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 (p=0.838).

Previously, an association of codon 54 (allele B) with vitiligo was observed in Turkish population where only two codons 54 and 57 were genotyped with smaller sample size (Onay et al., 2007); however, we found there was no association between vitiligo and *MBL2* structural and promoter polymorphisms. This is the first report that shows non-association of structural and promoter polymorphisms of *MBL2* gene with vitiligo. In conclusion, it can be considered that the structural and promoter polymorphisms in the *MBL2* gene may not confer a role in vitiligo susceptibility of Gujarat population.

4. Role of Interferon-γ (*IFN-γ*) gene intron 1 polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility:

Interferon-gamma (IFN-γ) is a key regulator of development and functions of the immune system. Genetic variability in intronic region of *IFN-γ* has been reported to be associated with its enhanced transcription thereby increasing the risk for several autoimmune diseases.

A) Genetic association of IFN-γ +874T/A (rs2430561) polymorphism with vitiligo patients and controls of Gujarat:

A single nucleotide T/A polymorphism at the 5' end of the CA repeat region in the first intron of the *IFN-γ* gene (+874 A/T polymorphism; rs2430561) has been correlated with the presence or absence of the microsatellite allele 2. Also, the presence of *IFN-γ* (+874 T^{hi}/A^{lo}) polymorphism creates a putative NF-κB binding site in intron1 and shows preferential binding to the T allele and correlates with high *IFN-γ* producer phenotype (Pravica et al., 2000). We genotyped *IFN-γ* noncoding +874T/A polymorphism (rs2430561) in 517 vitiligo patients and 881 ethnically, age-matched unaffected individuals by ARMS-PCR. Genotypes were also confirmed by sequencing of few random amplicons.

Intron1 +874T/A polymorphism of *IFN-γ* gene was not found to be associated with vitiligo patients as genotype frequencies did not differ between patients and controls (p=0.485). Also, there was no significant difference in allele frequencies of this polymorphism between patients and controls (p=0.274). Our results are in concordance with those of Namian et al. (2009) suggesting that +874T/A intron1 polymorphism of *IFN-γ* gene has no significant influence on vitiligo susceptibility. Therefore, we propose that *IFN-γ* +874T/A intron1 polymorphism is not a genetic risk factor for vitiligo susceptibility in Gujarat population.

B) Genetic association of IFN-γ CA microsatellite (rs3138557) polymorphism with vitiligo patients and controls of Gujarat:

The CA repeat microsatellite sequence in the first intron is polymorphic. Allele 2, with 12 CA repeats is associated with constitutively high IFN-γ production (*in vitro)* (Pravica et al., 2000). The genotyping of *IFN-γ* CA microsatellite is also being done in vitiligo patients and controls by high resolution melt curve (HRM) analysis using real time PCR and the results will be shown in the thesis.

C) Estimation of IFN-γ protein levels in patients and controls using ELISA:

The levels of IFN-γ in plasma samples of vitiigo patients and controls will be estimated using sandwich ELISA and the results will be shown in the thesis.

D) Establishment of genotype-phenotype correlation with vitiligo susceptibility:

To assess the effect of *IFN-γ* +874 T/A SNP and CA microsatellite, genotypes will be compared for the IFN-γ protein levels between patients and controls and thus genotype-phenotype correlation will be established for vitiligo susceptibility.

5. Role of Cytotoxic T-lymphocyte associated antigen-4 (*CTLA*-4) gene polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility:

CTLA-4 is a negative regulator of T-cell function, which is suggested to be involved in susceptibility to several autoimmune diseases including vitiligo. Genetic variability in *CTLA*-4 gene is reported to be associated with its altered levels thereby increasing the risk for several autoimmune diseases. We explored exon 1 +49A/G (rs231775) and 3' UTR CT60A/G (rs3087243) SNPs in *CTLA*-4 gene and correlated them with *CTLA*-4 transcript levels in vitiligo patients and controls of Gujarat.

A) Analysis of association between CTLA-4 exon 1 +49A/G (rs231775) polymorphism and susceptibility to vitiligo:

The single nucleotide polymorphism: A to G transition at position 49 (A49G) of exon 1 leads to an alanine to threonine amino acid substitution at codon 17 of the leader peptide (A17T) (Nistico et al., 1996). Interestingly, the G allele of *CTLA*-4 +49A/G SNP was reported to be involved in the altered intracellular transport of the CTLA-4 protein and its availability on the cell surface. We analyzed 347 vitiligo patients and 746 controls of Gujarat for *CTLA*-4 +49A/G polymorphism by PCR-RFLP. The genotype and allele frequencies for *CTLA*-4 +49A/G polymorphism did not differ significantly between vitiligo patients and controls (p=0.771; p=0.461 respectively) suggesting the non-association of +49A/G SNP with vitiligo susceptibility. Our results are in line with those of South Indian and Romanian populations (Deeba et al., 2010; Birlea et al., 2009).

B) Analysis of association between CTLA-4 3'UTR CT60A/G (rs3087243) polymorphism and susceptibility to vitiligo:

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 (sCTLA-4), suggesting its crucial role in autoimmune diseases (Ueda et al., 2003). We genotyped 437 vitiligo patients and 738 controls of Gujarat for *CTLA*-4 CT60A/G polymorphism by PCR-RFLP. The *CTLA*-4 CT60A/G polymorphism was found to be in significant

association with vitiligo patients, since the genotype and allele frequencies differed significantly between patients and controls ($p<0.0001$; $p<0.0001$ respectively). The frequency of CT60GG genotype was significantly higher in vitiligo patients as compared to controls suggesting that 3'UTR CT60A/G may be a genetic risk factor for vitiligo susceptibility in Gujarat population.

Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the two polymorphisms investigated in the *CTLA*-4 gene were in moderate LD association (+49A/G: CT60A/G; D' 0.64, $r^2=0.11$). A haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes did not differ between vitiligo patients and controls (global p-value = 0.123). However, the GG haplotype was more frequently observed in vitiligo patients and increased the risk of vitiligo by 1.2-fold [p=0.048; odds ratio (OR): 1.243; 95% confidence interval (CI): (1.001-1.543)].

C) Relative gene expression of flCTLA-4 and sCTLA-4 in patients and controls:

Analysis of fl*CTLA*-4 and s*CTLA*-4 mRNA expression showed significantly decreased expression in patients after normalization with *GAPDH* expression (p=0.007 and p=0.037 respectively). The $2^{-\Delta\Delta Cp}$ analysis showed approximately two fold change in the expression of fl*CTLA*-4 and s*CTLA*-4 mRNA expression in patients as compared to controls.

D) Establishment of genotype-phenotype correlation with vitiligo susceptibility:

We analyzed the mRNA expression of fl*CTLA*-4, s*CTLA*-4 based on the +49A/G and CT60A/G genotypes of 76 vitiligo patients and 83controls. The expression levels of fl*CTLA*-4 for AA, AG and GG genotypes of exon 1 +49A/G polymorphism did not differ significantly in vitiligo patients as compared to controls (p=0.122, p=0.320 and p=0.068 respectively). Also, s*CTLA*-4 expression did not differ for AA, AG and GG genotypes of exon 1 +49A/G polymorphism in vitiligo patients as compared to controls (p=0.877, p=0.437 and p=0.360 respectively). However, the expression levels of both fl*CTLA*-4 and s*CTLA*-4 were differed significantly for GG genotype of CT60A/G polymorphism in vitiligo patients as compared to controls (p=0.004 and p=0.005 respectively). The AA genotype did not differ for fl*CTLA*-4 and s*CTLA*-4 expression levels in patients and controls (p=0.343 and p=0.205 respectively).

Further, the expression levels of both fl*CTLA*-4 and s*CTLA*-4 were analyzed with respect to haplotypes generated from the two investigated polymorphisms of *CTLA*-4. Both fl*CTLA*-4 and s*CTLA*-4 expression levels were significantly differed and

associated with AG haplotypes in patients and controls (p=0.036 and p=0.020 respectively). Other, three haplotypes: AA, GA and GG did not differ with respect to fl*CTLA*-4 and s*CTLA*-4 expression levels in patients and controls (p=0.955 & p=0.152, p=0.476 & p=0.865, p=0.075 & p=0.992 respectively).

Ratio of sCTLA-4 and flCTLA-4 mRNA expression in vitiligo patients and controls:

The expression level of s*CTLA*-4 and fl*CTLA*-4 was also analyzed as ratio of s*CTLA*-4: fl*CTLA*-4 in vitiligo patients and controls. We could not detect any significant difference in the ratio of s*CTLA*-4 to fl*CTLA*-4 mRNA expression between patients and controls (p=0.346). However, AG genotype of exon 1 +49A/G polymorphism showed significant difference for the ratio of s*CTLA*-4 and fl*CTLA*-4 mRNA expression in patients and controls (p=0.049). Whereas the other two genotypes: AA and GG did not differ for the ratio of s*CTLA*-4 and fl*CTLA*-4 mRNA expression in patients and controls (p=0.668 and p=0.964). When ratio of s*CTLA*-4 and fl*CTLA*-4 mRNA expression for CT60A/G genotypes were compared in patients and controls GG genotype showed significantly decreased ratio (p=0.019), conversely the AA genotype did not differ significantly (p=0.096). Moreover, ratio of s*CTLA*-4 and fl*CTLA*-4 mRNA expression did not differ significantly for any of the haplotypes (AA, AG, GA, GG) in patients and controls (p=0.159, p=0.068, p=0.966 and p=0.585 respectively).

Our results suggest an association between CT60A/G polymorphism and susceptibility to vitiligo. 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 pathogenesis of vitiligo. CT60AA genotype did not modulate *CTLA*-4 mRNA expression suggesting patients harboring it may have other genetic factors involved in disease pathogenesis supporting the fact that vitiligo may have varied type of precipitating factors. Further, decreased s*CTLA*-4 mRNA levels in patients with haplotype AG (+49A:CT60G) reveals the positive correlation of CT60G in vitiligo pathogenesis.

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 polymorphism of the *CTLA*-4 gene influences both full length and soluble *CTLA*-4 mRNA levels in vitiligo patients, and thus this genotype-phenotype correlation of *CTLA*-4 supports the autoimmune pathogenesis of vitiligo.

6. Role of Melanocyte proliferating gene 1 (*MYG*1) promoter and structural polymorphisms and their genotype-phenotype correlation with vitiligo susceptibility:

*MYG*1 (Melanocyte proliferating gene 1 or Gamm1 or C12orf10, NM021640) is a ubiquitous nucleo-mitochondrial protein, involved in early developmental processes as well as in stress conditions (Kingo et al. 2006). Recently, an elevated expression of *MYG*1 mRNA has been shown in both uninvolved and involved skin in case of vitiligo (Kingo et al. 2006). The precise function of MYG1 in the development of vitiligo is not clear. However, up-regulation of several immune response-related genes after siRNA-mediated knockdown of *MYG*1 mRNA suggests that MYG1 may participate in pathways proposed by autoimmune theory of vitiligo pathogenesis. Alternatively, mitochondrially localized MYG1 can be involved in the regulation of altered metabolism and imbalance of antioxidants in vitiligo patients.

We explored -119C/G promoter polymorphism (rs1465073) and 11-12AA/GC structural polymorphism (rs1534284-rs1534283; Arg4Gln) in *MYG*1 gene to study their associations with vitiligo susceptibility and correlated them with *MYG*1 transcript levels in vitiligo patients and controls of Gujarat.

A) Genetic association of MYG1 -119C/G (rs1465073) polymorphism with vitiligo patients and controls of Gujarat:

The SNP rs1465073 is located 119 bp upstream of MYG1 translation start site (ATG) and designated as *MYG*1 promoter polymorphism (-119C/G). Previously, a strong association of -119G allele was observed in patients as compared to controls (Philips et al., 2010).

In the present study *MYG1* -119C/G promoter polymorphism (rs1465073) was genotyped in 662 patients and 724 controls. Genotype frequencies were significantly different between patients and controls suggesting a strong association of *MYG1* -119C/G promoter polymorphism with vitiligo susceptibility (p=0.001). Also, allele frequencies significantly differed between patients and controls for this polymorphism (p=0.002). Our results are in concordance with those of Philips et al., (2010) suggesting the important role of *MYG1* -119C/G promoter polymorphism in vitiligo susceptibility.

B) Genetic association of MYG1 11-12 AA/GC (rs1534284-rs1534283; Arg4Gln) polymorphism with vitiligo patients and controls of Gujarat:

This polymorphism involves nucleotides 11-12 (rs1534284-rs1534283; Arg4Gln) downstream from translation start site ATG. These nucleotides are coding second and third positions of amino acid four in the N-terminus of Myg1 protein (CAA and CGC, respectively). Amino acid four is part of a mitochondrial targeting signal (MTS) of Myg1 protein. The polymorphism is potentially functional, since it changes basic amino acid (Arginine) into polar and uncharged amino acid (Glutamine) which disturbs a common property of mitochondrial targeting sequence to form an amphiphilic helical structure that is essential for the effective transport of a mitochondrial protein.

MYG1 11-12 AA/GC (Arg4Gln) polymorphism was genotyped in 662 patients and 724 controls. This polymorphism was predominantly monogenic in patients and controls with only *MYG*1 GC (4Arg) alleles being present suggesting the non-association of this polymorphism with vitiligo susceptibility.

C) Relative gene expression of MYG1 in patients and controls:

*MYG*1 mRNA expression was assessed in 45 patients and 48 controls by real time PCR. Relative gene expression analysis of *MYG*1 showed significantly higher mRNA levels in patients as compared to controls ($p=0.004$) suggesting the crucial role of *MYG*1 in vitiligo susceptibility. The $2^{-\Delta\Delta Cp}$ analysis showed ~1 fold increased expression of *MYG*1 in patients as compared to controls.

D) Establishment of genotype-phenotype correlation with vitiligo susceptibility:

*MYG*1 mRNA levels in skin samples of healthy controls correlated with *MYG*1 promoter polymorphism -119C/G and subjects with homozygous -119G allele had significantly higher MYG1 mRNA levels than subjects with homozygous -119C allele (Philips et al., 2010). More samples will be analyzed to establish the genotype-phenotype correlation for the *MYG1* polymorphisms and the results will be shown in the thesis.

7. Role of NACHT leucine-rich repeat protein 1 (*NALP1*) gene promoter and structural polymorphisms in vitiligo pathogenesis:

*NALP*1, *(NLRP1)* encodes NACHT leucine-rich repeat protein 1, a key regulator of the innate immune system. *NALP*1 plays a role in cellular apoptosis, its over expression stimulates caspase mediated apoptosis in a variety of cell types. Variations in the *NALP*1 gene have been reported to confer risk for vitiligo and autoimmune disorders in Caucasian patients from the United Kingdom, the United States, and Romania (Jin et al., 2007).

A) Genotyping of NALP1 T/A (rs12150220), T/C (rs2670660) and G/A (rs6502867), polymorphisms in vitiligo patients and controls of Gujarat:

The T/A (rs12150220) is a non-synonymous coding SNP which substitutes leucine155 to histidine. The amino acid sequence, including Leu155, is highly conserved throughout primate evolution, suggesting that this region is critical for protein function. The T/C (rs2670660) SNP is present in *NALP*1 promoter region which is conserved in the human, chimpanzee, macaque, bush baby, cow, mouse and rat, suggesting that this variant is functionally significant. It alters predicted binding motifs for the transcription factors HMGA1 [HMG-I(Y)] and MYB. MYB regulates transcription during the differentiation, proliferation, and apoptosis of erythroid, myeloid, and lymphoid cell lineages. The G/A (rs6502867) SNP is located in an intron towards the 3' end of the *NALP*1 gene. The Romanian study confirms genetic association of generalized vitiligo with *NALP*1 SNP rs6502867 and SNP rs2670660. Genotyping of *NALP1* G/A (rs6502867), T/C (rs2670660), T/A (rs12150220) SNPs is being done in vitiligo patients and controls of Gujarat and the results will be shown in the thesis.

B) Relative gene expression of NALP1 in patients and controls:

NALP1 transcript levels will be measured in vitiligo patients and controls with *GAPDH* as a reference gene using real time PCR and the results will be shown in the thesis.

C.) Establishment of genotype-phenotype correlation with vitiligo susceptibility:

To assess the effect of *NALP*1 G/A, T/C and T/A polymorphisms, genotypes will be compared with the *NALP*1 mRNA levels between patients and controls and thus genotype-phenotype correlation will be established for vitiligo susceptibility.

8. Determination of $CD4^+/CD8^+$ ratio and estimation of $CD4^+CD25^+$FoxP3 T-regulatory cells (Tregs) in vitiligo patients and controls:

A) Determination of $CD4^+/CD8^+$ ratio *in vitiligo patients and controls of Gujarat:*

High frequencies of melanocyte-reactive cytotoxic T cells in the peripheral blood of vitiligo patients, peri-lesional T-cell infiltration and melanocyte loss *in situ* suggest the important role of cellular autoimmunity in the pathogenesis of this disease. In most vitiligo patients the balance of cytotoxic/suppressor and helper/inducer T-cells in peripheral blood is disturbed which might lead to predominance of T-cell subtypes. The ratio of $CD4^+/CD8^+$ cells will be monitored by FACS analysis in vitiligo patients and controls of Gujarat population and the results will be shown in the thesis.

B) Estimation of $CD4^+CD25^+$FoxP3 T-regulatory cells (Tregs) in vitiligo patients and controls of Gujarat:

Regulatory T cells (Tregs) are critical for the maintenance of immune cell homeostasis. Specifically, Treg cells maintain order in the immune system by enforcing a dominant negative regulation on other immune cells. CD4+CD25+ Tregs is an important component of immune system in controlling autoimmunity. These naturally occurring T cells can control actively and dominantly the activation and function of autoreactive T cells that have escaped from the thymus and can prevent development of the autoimmune disease. The FoxP3 is a special marker for Treg cells; it has been identified as a master gene for the cell-lineage commitment, development and function of Treg cells.

In the present study, vitiligo patients exhibited decreased s*CTLA*-4 and fl*CTLA*-4 transcript levels. Recently, Gerold et al., (2011) have also shown decreased s*CTLA*-4 levels in Type-1 diabetic condition suggesting that lower s*CTLA*-4 expression may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. Hence, CD4+CD25+ Tregs will be monitored in vitiligo patients and controls by FACS analysis and the results will be shown in the thesis.

Conclusion:

The present study has made an attempt to understand the pathogenesis of vitiligo in Gujarat vitiligo patients by assessing antimelanocyte antibody levels and role of selected candidate genes involved in autoimmune pathogenesis of vitiligo. Significant increase in antimelanocyte antibody levels in vitiligo patients supports the involvement of autoimmunity in vitiligo pathogenesis. Our results on genetic study suggests that candidate genes: *PTPN22*, *ACE*, *MBL2*, *IFN-γ* are not playing important role in vitiligo pathogenesis. However, significant association of polymorphisms of *CTLA-4* and *MYG1* genes and their altered mRNA expression suggest their crucial role in vitiligo susceptibility.

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