SYNOPSIS

Introduction

Vitiligo is an idiopathic, acquired, circumscribed hypomelanotic skin disorder, characterized by milky white patches of different size and shape. The clinical hallmark of vitiligo is loss of melanin pigment due to decreased number of functional melanocytes or their complete absence in the lesional skin. Based on a few dermatological outpatient records, the prevalence of vitiligo is found to be 0.5 - 2.5% in India (Handa and Kaur 1999). Though vitiligo has been extensively addressed in the past six decades, its etiology is still being debated (Taieb, 2000). Oxidative stress hypothesis suggests that impaired redox status of the epidermal melanin unit is the primary defect leading to inappropriate immune response resulting in melanocyte destruction. Epidermal H_2O_2 generation in vitiliginous patients could be due to several factors (Schallreuter *et al.*, 1996; Rokos *et al.*, 2002) including defective recycling of tetrahydrobiopterin in vitiligo epidermis. Superoxide dismutases scavenge superoxide radicals and reduce its toxicity by converting it to H_2O_2 (Schallreuter *et al.*, 1991). The increased activity of superoxide dismutases with unaltered or decreased activity in catalase and glutathione peroxidase leads to accumulation of H_2O_2 which may be toxic to the melanocytes. Impairment in the systemic antioxidant system is also observed in our study (Agrawal *et al.*, 2004; Shajil and Begum, 2006). Thus our biochemical studies suggest that the melanocyte damage in vitiligo may be linked with generalized oxidative stress.

Although etiology of vitiligo remains obscure, autoimmunity has also been suggested to play a major role in the pathogenesis of the disease (Kemp *et al.*, 2001). The autoimmune hypothesis is based on the fact that vitiligo is frequently associated with other autoimmune diseases. Anti-melanocyte and anti-keratinocyte antibodies are present in circulation of most of the vitiliginous patients (Cui *et al.*, 1992). Moreover, auto-reactive cytotoxic T lymphocytes (CTLs) are detected in both the peripheral blood and perilesional skin of vitiligo patients (Palermo *et al.*, 2001). Cytokines are small immune-regulatory molecules which can generate an inappropriate immune response due to their imbalance. Morreti *et al.*, (2002) have shown an imbalance of cytokines in the skin of vitiligo patients suggesting their role in autoimmunity. TNF-α is a cytokine which is involved in the regulation of immune cells and overproduction of TNF-α has been implicated in a variety of human diseases including autoimmune disorders and cancer (Locksley *et al.*, 2001). Guha *et al.*, (2000) have shown increased levels of tumor necrosis factor-α (TNF-α) due to

enhanced *TNFA* gene transcription, mediated by ROS via activation of transcription factors such as nuclear factor kB (NF-kB) and activating protein-1 (AP-1). TNF-β (Lymphotoxin-α) is produced by activated lymphocytes and carries out most of the activities of the structurally related molecule i.e., TNF-α.

The importance of genetic factors for vitiligo susceptibility is evident by reports of significant familial association (Bhatia *et al.*, 1992; Nordlund, 1997). About 20% of vitiligo patients have at least one first-degree relative affected (Bhatia *et al.*, 1992; Nath *et al.*, 1994) indicating that genetic predisposition may be involved in vitiligo susceptibility. Our studies also suggest that 22% of Gujarat vitiligo patients exhibit positive family history and 14% patients have at least one first-degree relative affected (Shajil *et al.*, 2006). The inheritance pattern of vitiligo does not follow the simple Mendelian pattern and its mode of heredity suggests that it is a polygenic disease. Several candidate genes including major histocompatibility complex (*MHC)*, autoimmune susceptibility loci (*AIS*1, *AIS*2, *AIS*3), autoimmune regulator (*AIRE*), angiotensin converting enzyme (*ACE)*, catalase (*CAT)*, cytotoxic T-lymphocyte antigen-4 (*CTLA-4*), catechol O-methyl transferase (*COMT)*, mannose binding lectin 2 (*MBL2*), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), and NACHT- leucine-rich-repeat protein 1 (*NALP1*) are reported for vitiligo susceptibility (Spritz, 2008; Fain *et al.*, 2003). In order to explore the genetic susceptibility, systematic study of each gene governing oxidative stress and regulation of autoimmunity is essential.

Most of the studies on vitiligo are done in western countries; however clinical and genetic studies on Indian population are scarce. Since environmental factors could also account for the origin of autoimmunity/reactive oxygen species (ROS) in the susceptible patients, clinical studies on vitiligo in Indian patients addressing the oxidative stress and autoimmune hypotheses is required. Hence, a systematic study was attempted to investigate the role of superoxide dismutases, tumor necrosis factor-α & -β in vitiligo susceptibility of Gujarat population.

Objectives of the present study:

1. Role of superoxide dismutase 1 (SOD1) in vitiligo susceptibility of Gujarat population.
2. Role of superoxide dismutase 2 (SOD2) in vitiligo susceptibility of Gujarat population.
3. Role of superoxide dismutase 3 (SOD3) in vitiligo susceptibility of Gujarat population.
4. Role of tumor necrosis factor-α (TNF-α) in vitiligo susceptibility of Gujarat population.
5. Role of tumor necrosis factor-β (TNF-β) in vitiligo susceptibility of Gujarat population.

The study group included 1005 vitiligo patients comprised of 450 males and 555 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India. The diagnosis of vitiligo was clinically based on presence of depigmented patches on the skin and patients had no other associated autoimmune diseases. The study involved generalized vitiligo patients including acrofacial vitiligo and vitiligo universalis. A total of 1710 ethnically and sex-matched unaffected individuals were included in this study. The control group comprised of 730 males and 980 females. None of the healthy individuals had any evidence of vitiligo and autoimmune diseases. The importance of the study was explained to all the participants and written consent was obtained from all patients and controls.

1. Role of superoxide dismutase 1 (SOD1) in vitiligo susceptibility of Gujarat population:

The *SOD1* gene is localized to chromosome 21q22 in humans. *SOD1* is a constitutively expressed gene and its gene product is a dimeric Cu^{++} and Zn^{++} containing enzyme of 32 kDa.

Estimation of lipid peroxidation (LPO) levels to assess oxidative stress in vitiligo patients and control population:

Oxidative stress has been suggested to be a primary cause of vitiligo and high lipid peroxidation levels serve as marker for oxidative stress. Hence, LPO levels were estimated in 950 vitiligo patients and 1650 controls. Vitiligo patients showed a

significant ($p<0.0001$) increase in LPO levels as compared to controls suggesting increased oxidative stress in vitiligo patients.

Estimation of Superoxide dismutase 1 activity in Gujarat vitiligo patients compared to controls:

The estimation of SOD1 activity in erythrocytes was carried out in 950 vitiligo patients and 1650 controls. The SOD1 activity was significantly ($p=0.001$) higher in vitiligo patients as compared to controls which suggests increased H_2O_2 production in patients which in turn may be toxic to melanocytes.

Estimation of* SOD1 *protein levels in vitiligo patients compared to controls:

Western blot analysis was performed to explore whether the increased SOD1 activity in patients compared to controls is due to increased expression of SOD1 protein. We have monitored SOD1 levels in 50 control subjects and 50 vitiligo patients. After normalizing the results with respect to GAPDH internal control, our densitometric analysis showed that there is no significant change in SOD1 protein levels in vitiligo patients compared to controls ($p=0.979$) suggesting that increased SOD1 activity is not due to increased SOD1 protein level. Thus, there may be mutations associated in the exonic regions of this gene which may enhance the activity of SOD1.

Genetic association of C/T (Ile40Thr) single nucleotide polymorphism in exon 2 of SOD1 gene in vitiligo patients and controls:

Our biochemical studies showed that systemic oxidative stress in vitiligo patients is due to alteration in the antioxidant system in vitiligo patients compared to controls. SOD1 activity was found to be significantly increased in vitiligo patients as compared to controls. We have attempted to explore whether increased activity of this enzyme is linked to one of the reported *SOD*1 SNP (Bergholdt *et al.*, 2005), C/T exon 2 (Ile40Thr) (rs1804450) in 165 vitiligo patients and 196 controls. The genotyping was done by RFLP analysis of PCR amplified genomic fragments using restriction endonuclease *Acu*I. The allele frequencies of C/T SNP did not differ significantly between control and patient population ($p=1.0$). When the observed control and patient genotype frequencies were compared, it did not show significant change suggesting that there is no association of the *SOD*1 C/T exon 2 SNP with vitiligo. Our

results suggest that this polymorphism seems to be uninformative in Gujarat population.

***SOD*1 gene scanning for novel genetic variations using High Resolution Melt Curve (HRM) Analysis:**

Total 62 vitiligo patients and 30 controls were analyzed for any genetic variants in SOD1 gene. SOD1 gene has five exons and four introns. Each exon of SOD1 amplified was subjected to HRM analysis using Real time PCR. All exons and their exon – intron boundries were amplified except exon 5 which was splitted into two fragments for amplification due to its large size. However, we could not find different groups generated by HRM analysis on difference plot and hence products from the single group were sequenced to confirm the results. Nevertheless, no mutations/SNPs detected based on BLAST search with Human SOD1 wild type sequence.

2. Role of Superoxide dismutase 2 (SOD2) in vitiligo susceptibility of Gujarat population:

The *SOD2* gene is localized to chromosome 6 q25 in humans. Its gene product is a mitochondrial tetrameric Mn^{++} containing enzyme of 89 kDa. The *SOD2* gene is composed of 5 exons and 4 introns. SOD2 (MnSOD) is involved in controlling dioxygen toxicity in the mitochondria, an organelle of extreme oxidative load. MnSOD dismutates O_2•- to H_2O_2, which is further detoxified by GPX1 in mitochondria. If not be quenched, H_2O_2 will be converted to the more toxic hydroxyl radical (•OH). Hence, study of the effect of the *SOD2* gene polymorphisms might provide information on the role played by oxidative stress in vitiligo.

Estimation of Superoxide dismutase 2 activity in Gujarat vitiligo patients compared to controls:

Estimation of SOD2 activity in leucocytes was carried out in 170 vitiligo patients and 150 controls and its activity was found to be significantly ($p=0.013$) higher in vitiligo patients as compared to controls.

Genetic association of C/T (*Ala16Val*) single nucleotide polymorphism in exon 2 of SOD2 gene in vitiligo patients of Gujarat and controls:

An Alanine (GCT) to Valine (GTT) substitution (rs4880) at position-9 in the signal peptide of human SOD2 is reported to change the conformation of the mitochondrial targeting sequence of the enzyme. This substitution may lead to misdirected intracellular trafficking, followed by changes in SOD2 activity in the mitochondria which can compromise the ability to neutralize superoxide radicals in the cell. Studies have suggested basal levels of SOD2 activity are the highest for Ala/Ala, followed by Ala/Val, and then Val/Val. The experiment on genotyping of the *SOD2 C/T (*Ala9Val) SNP in vitiligo patients and controls of Gujarat has been attempted and the results will be shown in the thesis.

Genetic association of C/T (Ile58Thr) single nucleotide polymorphism in exon 3 of SOD2 gene in vitiligo patients of Gujarat and controls:

The polymorphism of the *SOD2* gene, Ile58Thr (rs35289490) in exon 3, affects the stability of the *SOD2* tetramer and reduces the activity of the enzyme. Cells that over express the Ile58 allele exhibit higher SOD2 activity than cells that over express the Thr58 allele. The genotyping of the *SOD2* C/T (Ile58Thr) SNP is being done in vitiligo patients and controls of Gujarat and the results will be shown in the thesis.

3. Role of superoxide dismutase 3 (SOD3) in vitiligo susceptibility of Gujarat population:

EC-SOD (*SOD3*) plays a protective role against increased oxidative stress in the extracellular space, where it is the major extracellular protein capable of removing superoxide. The *SOD3* gene is located on chromosome 4 (4p16.3-q21) and comprises three exons and two introns.

Estimation of superoxide dismutase 3 activity in vitiligo patients compared to controls:

In the present study significant increase in SOD3 activity as well as SOD3 specific activity (EC SOD) was observed in plasma of 445 vitiligo patients compared to 550 controls. This increase in SOD3 activity was found to be 1.7 fold higher in vitiligo patients compared to controls ($p=0.003$). Thus increased activity of EC SOD in vitiligo patients could enhance the systemic production of H_2O_2 which in turn might lead to high oxidative stress in patients.

Genetic association of* C/G *(*Arg231Gly*) single nucleotide polymorphism in exon 3 of SOD3 gene in vitiligo patients and controls:

The C/G SNP (rs1799895) present in *SOD3* gene which substitutes Arginine to Glycine at codon 231 inhibits ionic interaction between heparin and SOD3 which in turn results in 10 fold increase in SOD3 concentration in plasma (Sandstrom *et al.*, 1994). This polymorphism was genotyped by RFLP analysis of PCR amplified genomic fragments using restriction endonuclease *Mwo*I in 548 controls and 455 patients. From genotype analysis, we have confirmed that there is significant (p=0.001) association of C/G (Arg231Gly) SNP of *SOD3* in Gujarat vitiligo patients compared to controls. The allele frequencies of C/G SNP differed significantly between control and patient population (p=0.001). The frequency of GG genotype was higher in vitiligo patients as compared to controls which may lead to increased SOD3 activity in respective vitiligo individuals. Thus, our biochemical studies in combination with *SOD3* polymorphism analysis suggest that oxidative stress may play a major role in pathogenesis of vitiligo in Gujarat population.

Genetic association of* A/G *(*Ala40Thr*) single nucleotide polymorphism in exon 1 of SOD3 gene in vitiligo patients of Gujarat and controls:

The A/G SNP (rs2536512) present in *SOD3* gene which substitutes Alanine to Threonine polymorphism is located in the amino terminal domain, and may be crucial for the tetramerization of the enzyme. It is unclear whether the Ala40Thr polymorphism affects directly some functional properties of EC-SOD. Our biochemical studies showed that SOD3 activity was found to be significantly increased in vitiligo patients compared to controls. We have attempted to explore whether increased activity of this enzyme is linked to the well documented single nucleotide polymorphism A/G *(*Ala40Thr) in *SOD3* gene. The genotyping of the A/G *(*Ala40Thr) SNP is being done in vitiligo patients and controls of Gujarat and the results will be shown in the thesis.

4. Role of tumor necrosis factor-α (TNF-α) in vitiligo susceptibility of Gujarat population:

TNF-α is a paracrine inhibitor of melanocytes which is secreted by neighbouring keratinocytes and it plays an important role in vitiligo pathogenesis. In

the epidermal melanin unit of epidermis, a melanocyte is in close interaction with ~32 keratinocytes. Close relationship between these two cell types is important as keratinocyte-derived cytokines act on melanocytes via specific receptors and help for melanocyte survival and differentiation (Moretti *et al.*, 2002). TNF-α is also produced by activated monocytes in response to a variety of signals including stress response, phorbol esters, cytokines, endotoxin, and substrate adherence (Yao *et al.*, 1997). *TNFA* gene expression has also been shown to be increased in response to oxidative stress.

Several single nucleotide polymorphisms (SNPs) have been identified in the human TNF-α gene promoter. The polymorphisms at positions -238, -308, -857, -863 and -1031 at the promoter lead to a higher rate of *TNFA* gene transcription than the wild type genotype in *in vitro* expression studies. Hence we have made an attempt to study the role of these polymorphisms in vitiligo susceptibility.

Genotyping of TNFA -238 polymorphism (rs361525) in vitiligo patients and controls:

Polymorphism G/A substitution in promoter region of *TNFA* gene at -238 position was genotyped by RFLP analysis of PCR amplified genomic fragments using *BamH*I in 334 vitiligo patients and 616 controls of Gujarat. The allele frequencies of G/A SNP differ significantly between control and patient population ($p<0.0001$). Genotype frequencies significantly differed between patients and controls ($p<0.0001$) suggesting that there is strong association of the *TNFA* -238G/A SNP with vitiligo susceptibility.

Genotyping of TNFA -308 polymorphism (rs1800629) in vitiligo patients and controls:

Polymorphism G/A substitution in promoter region of *TNFA* gene at -308 position was genotyped by RFLP analysis of PCR amplified genomic fragments using *Nco*I in 379 vitiligo patients and 762 controls of Gujarat. The allele frequencies of G/A SNP differ significantly between control and patient population ($p<0.0001$). Genotype frequencies significantly differed between patients and controls ($p<0.0001$) suggesting that there is strong association of the *TNFA* -308G/A SNP with vitiligo susceptibility.

Genotyping of TNFA -857 polymorphism (rs1799724) in vitiligo patients and controls:

Polymorphism C/T substitution in promoter region of *TNFA* gene at -857 position was genotyped by RFLP analysis of PCR amplified genomic fragments using *Tai*I in 357 vitiligo patients and 644 controls of Gujarat. The allele frequencies of C/T SNP differ significantly between control and patient population ($p<0.0001$). Genotype frequencies significantly differed between patients and controls ($p<0.0001$) suggesting that there is strong association of the *TNFA* -857C/T SNP with vitiligo susceptibility.

Genotyping of TNFA -863 polymorphism (rs1800630) in vitiligo patients and controls:

Polymorphism studied was C/A substitution in promoter region of *TNFA* gene at -863 position and was genotyped by RFLP analysis of PCR amplified genomic fragments using *Tai*I in 362 vitiligo patients and 697 controls of Gujarat. The allele frequencies of C/A SNP differ significantly between control and patient population ($p<0.0001$). Genotype frequencies significantly differed between patients and controls ($p<0.0001$) suggesting that there is strong association of the *TNFA* -863C/A SNP with vitiligo susceptibility.

Genotyping of TNFA -1031 polymorphism (rs1799964) in vitiligo patients and controls:

Polymorphism C/T substitution in promoter region of *TNFA* gene at -1031 position was genotyped by RFLP analysis of PCR amplified genomic fragments using *Bbs*I in 324 vitiligo patients and 651 controls of Gujarat. The allele frequencies of C/T SNP differ significantly between control and patient population ($p<0.0001$). Genotype frequencies significantly differed between patients and controls ($p<0.0001$) suggesting that there is strong association of the *TNFA* -1031C/T SNP with vitiligo susceptibility.

TNF-α *serum levels in vitiligo patients and controls:*

A total of 86 vitiligo patients and 90 controls were investigated for TNF-α serum levels by Sandwich ELISA. Higher TNF-α serum levels were found in vitiligo patients as compared to controls (p=0.001).

Gene expression analysis of TNFA in vitiligo patients and controls:

TNFA mRNA expression was assessed in 110 patients and 140 controls by real time PCR. Significant increase in expression of *TNFA* was observed in vitiligo patients compared to controls after normalization with *GAPDH* expression (p=0.001). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.5 fold change in *TNF-α* expression in patients as compared to controls.

Genotype-phenotype correlations for TNF-α in vitiligo patients and controls:

We analyzed the mRNA expression of *TNFA* based on the five promoter polymorphisms genotypes in 110 vitiligo patients and 140 controls. The expression levels of *TNFA* for AA genotypes of -238G/A polymorphism significantly differed in vitiligo patients as compared to controls (p=0.006). However, GG and GA genotypes did not differ (p=0.563; p=0.139 respectively) in *TNFA* mRNA levels as compared to controls. Also, *TNFA* expression did not differ for GG and GA genotypes of -308G/A polymorphism in vitiligo patients as compared to controls (p=0.625; p=0.272 respectively) whereas AA genotypes showed significantly higher expression of *TNF-α* in patients as compared to controls (p=0.009). However, the expression levels of *TNFA* differed significantly for TT genotype of -857C/T polymorphism in vitiligo patients as compared to controls (p<0.0001) whereas CC and CT genotypes did not differ for *TNFA* expression (p=0.273; p=0.919 respectively). The CA genotype of -863C/A polymorphism differed significantly for *TNFA* expression between patients and controls (*p*=0.008), whereas CC and AA genotypes did not differ (p=0.327; p=0.064 respectively) in patients as compared to controls. *TNFA* expression did not differ for CC and CT genotypes of -1031C/T polymorphism in vitiligo patients as compared to controls (p=0.764; p=0.951 respectively) whereas TT genotypes showed significantly higher expression of *TNFA* in patients as compared to controls (p=0.034).

5. Role of tumor necrosis factor-β (TNF-β) in vitiligo susceptibility of Gujarat population:

TNF-α and TNF-β are the important members of TNF family. The *TNFA* and *TNFB* genes are located adjacent to each other in the major histocompatibility complex class III region on chromosome 6p21.3. TNF-β that is encoded by lymphotoxin- gene (LTA) regulates adhesion molecules and IL-6. Several variants of *TNFB* have been described, including +252G/A polymorphism in the first intron. This SNP is in strong linkage disequilibrium with another polymorphism, Thr26Asn that results in the substitution of a threonine residue with an asparagine residue at codon 60 in exon 3.

Genetic association of +252 G/A (rs909253) single nucleotide polymorphism in TNFB gene in vitiligo patients and controls:

The G/A SNP is present in intron 1 of *TNFB* gene which was genotyped by RFLP analysis of PCR amplified genomic fragments using restriction endonuclease *Nco*I in 592 controls and 493 patients. *TNFB* +252G is associated with higher TNF-β production. Genotype frequencies significantly differed between patients and controls (p=0.007) suggesting that there is strong association of the *TNFB* +252G/A SNP with vitiligo susceptibility. The allele frequencies of G/A SNP also differed significantly between control and patient population (p=0.002). Our results showed higher frequency of GG genotypes in vitiligo patients as compared to controls which may lead to increased *TNF*-β levels in patients.

Genetic association of Thr26Asn A/C (rs1041981) single nucleotide polymorphism in exon 3 of TNFB gene in vitiligo patients and controls:

The Thr26Asn polymorphism in the *TNFB* gene is in linkage disequilibrium with the +252 G/A intron 1 polymorphism. *In vitro* experiments have shown that the Thr26Asn polymorphism is associated with a two fold increase in the induction of cell-adhesion molecules in the vascular smooth muscle cells of human coronary arteries. The genotyping of the *TNFB* A/C (Thr26Asn) SNP is being done in vitiligo patients and controls of Gujarat and the results will be shown in the thesis.

Gene expression analysis of TNFB in vitiligo patients and controls:

Gene expression analysis for *TNFB* will be done in patients and controls by real time PCR, using *GAPDH* as an internal control and the results will be shown in the thesis.

Genotype-phenotype correlation for TNFB in vitiligo patients and controls:

To assess the effect of the +252 G/A and Thr26Asn A/C polymorphisms in *TNFB* gene, genotypes will be compared with the corresponding transcript levels between patients and controls and thus, genotype-phenotype correlation will be established for vitiligo susceptibility.

Conclusion:

The above study shows significant increase in the activity of all three types of SODs in vitiligo patients which indicates that melanocyte damage in vitiligo may be linked with generalized oxidative stress. Also our SOD1 western results suggest that increased SOD1 activity in vitiligo patients is not due to increase in the SOD1 protein levels suggesting for the presence of novel SNP/s in the *SOD* gene, which could enhance the SOD1 activity in Gujarat vitiligo patients. Our genetic polymorphism studies suggest that *SOD3* 760C/G SNP is strongly associated with vitiligo patients suggesting its role in oxidative stress related pathogenesis of vitiligo. Our results also suggest that early onset vitiligo patients (<3 months duration of vitiligo) exhibit higher oxidative stress and lower antimelanocyte antibody levels as compared to patients with >3 months duration of vitiligo suggesting the primary role of oxidative stress in vitiligo pathogenesis which could further induce autoimmune response in these patients.

TNFA promoter polymorphisms: -238 (rs361525), -308 (rs1800629), -857 (rs1799724), -863(rs1800630) and -1031 (rs1799964) and *TNFB* +252G/A SNP were found to be significantly associated with vitiligo susceptibility in Gujarat population. In addition, a higher expression of *TNFA* gene was detected in patients compared to controls. Overall, our results suggest that ROS may increase the TNF-α levels in patients, and thus oxidative stress coupled to increased *TNF*-α expression may contribute for vitiligo pathogenesis.

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