CHAPTER III

ROLE OF SUPEROXIDE DISMUTASE 2 (SOD2) IN VITILIGO SUSCEPTIBILITY

3.1 INTRODUCTION

Aerobic organisms have several mechanisms to protect from harmful reactive oxygen species (ROS). The principle cellular anti-oxidants are the superoxide dismutase family (SOD, E.C. 1.15.1.1). These enzymes dismutate superoxide into hydrogen peroxide which is further detoxified by other cellular defences such as glutathione peroxidase and catalase. Superoxide and its products have been implicated in a wide range of diseases including cancer, inflammation, neurodegenerative diseases, diabetes and aging. The SOD family has three members, two of which are Cu-Zn type- the extracellular (EC) SOD3 and the cytoplasmic SOD1. The other member is the mitochondrial Mn type SOD2.

SOD2 is involved in controlling dioxygen toxicity in the mitochondria, an organelle of extreme oxidative load (Fridovich *et al.*, 1995). Mitochondria are the primary source of endogenous ROS, since approximately 2% of NADH-derived electrons are transferred to molecular oxygen rather than to ubiquinone by Complex I of the respiratory chain, and this transfer results in the formation of the highly reactive superoxide anion (Turrens and Boveris, 1980). Superoxide is also a by-product of electron transfer by Complex III. Mitochondrial manganese superoxide dismutase is a critical antioxidant protein that reduces superoxide radical to hydrogen peroxide (Klug *et al.*, 1972). Within mitochondria, SOD2 provides a major defence system against oxidative damage by reactive oxygen species generated from mitochondrial electron transport chain during ATP synthesis process.

SOD2 protein is encoded by the nuclear genome and *SOD2* gene is present on chromosome 6q25. The *SOD2* gene consists of 5 exons interrupted by 4 introns with typical splice junctions (Wan *et al.*, 1994). *SOD2* mRNA translates in cytosol and translocates into the mitochondria via an N-terminal signal peptide, which plays a key role in targeting the enzyme to mitochondria (Church *et al.*, 1990; Church *et al.*, 1993). SOD2 docks in the mitochondrial matrix as a homotetramer of subunit mass 23 kDa. Amino acid sequences of SOD2 among species are highly conserved, and their homologies between human and rodent and between rat and mouse are 93% and 96%, respectively (Ho *et al.*, 1998; DiSilvestre *et al.*, 1995). This conservation may be critical to maintain its enzymatic function.

SOD2 is regarded as one of the key enzymes involving the anti-oxidation in the clinical disorders. Several studies have suggested the importance of SOD2 in clinical disorders. The SOD2 has been known to be associated with cancers (Li *et al.*, 2005), diabetic nephropathy (Nomiyama *et al.*, 2003), dilated cardiomyopathy (Shimoda-Matsubayashi *et al.*, 1996; Shimoda-Matsubayashi *et al.*, 1997; Li *et al.*, 1995; Huang *et al.*, 2001), neurodegenerative diseases (Hinerfeld *et al.*, 2004) and brain ischemia (Kim *et al.*, 2002).

An alteration in the antioxidant system, with a significant reduction in catalase activity has been demonstrated in both lesional and non-lesional epidermis of vitiligo patients (Schallreuter *et al.*, 1991) as well as in melanocytes derived from patients (Maresca *et al.*, 1997). Antioxidant imbalance in peripheral blood mononuclear cells of active vitiligo patients is also observed. An increased intracellular production of ROS appeared to be due to mitochondrial impairment (Dell'Anna *et al.*, 2001). These findings support the concept of a possible systemic oxidative stress in vitiligo. Sravani *et al.*, 2009 demonstrated significant increase in the levels of SOD and low catalase levels in vitiliginous and non vitiliginous skin of patient group compared to the control group suggesting high oxidative stress in vitiligo patients. Jain *et al.*, 2011 reported significantly higher SOD activity in blood of both active and stable vitiligo patients as compared to controls. Our previous reports have also shown an imbalance in the antioxidant enzyme system with increased activity of erythrocyte SOD in vitiligo patients from Gujarat suggesting high oxidative stress in patients (Agrawal *et al.*, 2004; Shajil and Begum, 2006).

Nevertheless, the exact genetic defects in antioxidant enzymes that lead to their altered levels/activity leading to oxidative stress mediated damage of melanocytes in vitiligo are still unknown. Also there are no reports on *SOD2* polymorphisms in vititligo pathogenesis till date. Therefore, addressing the genetic polymorphisms in *SOD2* gene is essential to understand its role in vitiligo pathogenesis.
We have addressed four *SOD2* polymorphisms in the present study.

Val16Ala (T/C; rs4880): The SOD2 signal sequence is essential for correct transport activity of proteins by mitochondria (Nelson and Cox 2005). A polymorphism in the second exon of the *SOD2* gene results in an alanine-to-valine change at amino acid position 16 (Val16Ala) (Shimoda-Matsubayashi *et al.*, 1996). This polymorphism

may change the protein conformation and mitochondrial transport of SOD2 (Shimoda-Matsubayashi *et al.*, 1996; Rosenblum *et al.*, 1996). As a result, the alanine-containing protein shows normal transport and generates 30-40% more active enzyme than does the valine form of the enzyme (Sutton *et al.*, 2003). Moreover, the Ala variant of the mitochondrial targetting signal (MTS) has been shown to improve the processing efficiency, resulting in increased protein tetramers of SOD2 in the mitochondria (Sutton *et al.*, 2003). Clinically different genetic variants were associated with different diseases, such as Ala/Ala with increased risk for cancers (Li *et al.*,2005; Mikhak *et al.*,2008) and Val/Val homozygosity with the development of diabetic nephropathy (Nomiyama *et al.*,2003; Liu *et al.*,2009). Fujimoto *et al.* (2008) found an association between the alanine variant of the signal peptide and increased mitochondrial SOD2 activity, which protects macrophages from the oxLDL-induced apoptosis and reduces the risk of acute coronary syndrome and cardiovascular diseases.

Thr58Ile (C/T; rs35289490): A polymorphic locus, amino acid codon +58 (T5777C, Ile or Thr allele) located in the exon 3, is related to the potency of SOD2. In Thr58Ile polymorphism, SOD2 with threonine has a weaker antioxidant activity than that with isoleucine (Ho *et al.*, 1998). The polymorphism, Thr58Ile, affects the stability of protein tetrameric interface and reduces the biological activity of SOD2 (Borgstahl *et al.*, 1996; Ho and Crapo, 1988). The +58Ile protein form showed a three-fold higher activity than the +58Thr form (Zhang *et al.*, 1999). Besides, from the molecular viewpoint, the unique genetic expression of Ile/Ile homozygosity at codon +58 implies the conserved functional domain of SOD2 structure. The *SOD2* gene with T at nucleotide position 5777 encodes a native form of SOD2, which has a stable tetrameric interface. However, *SOD2* gene with a nucleotide sequence 5777C encodes a mutant form that has increased thermal instability and accelerated thermal inactivation (Borgstahi 1992; Borgstahl *et al.*, 1996).

Both the above polymorphisms with substituted amino acids altered the SOD2 enzyme activity (Martin *et al.*,2005; Zhang *et al.*,1999; Sutton *et al.*,2003) resulting in altered anti-oxidative metabolism. In addition, two non-synonymous SNPs [Leu84Phe (C/T; rs11575993) and Ile82Thr (T/C; rs1141718)] in *SOD2* gene have also been

addressed in the present study. However, the functional significance of these two SNPs has not been reported.

In the present study, we have made an attempt to understand the role of SOD2 in vitiligo pathogenesis and the objectives of this study were:

i) To determine whether the following four polymorphisms of *SOD2* are associated with vitiligo susceptibility and modulate SOD2 activity.
   a) Val16Ala (T/C; rs4880)
   b) Thr58Ile (C/T; rs35289490)
   c) Ile82Thr (T/C; rs1141718)
   d) Leu84Phe (C/T; rs11575993)

ii) To measure and compare *SOD2* transcript levels and activity in patients with vitiligo and in unaffected controls.

iii) To correlate *SOD2* polymorphisms, expression levels and its activity with progression of the disease.

3.2 MATERIALS AND METHODS

3.2.1 Study Subjects:

The study group included 520 vitiligo patients comprised of 228 males and 292 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. A total of seven hundred and fifty ethnically, sex-matched unaffected individuals comprised of 338 males and 412 females were included as controls in this study (Table 1). None of the healthy individuals had any evidence of vitiligo and any other autoimmune disease. The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

3.2.2 Blood collection and genomic DNA preparation:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analysis.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	Controls
	(n = 520)	(n = 750)
Average age (mean age ± SD)	32.45 ± 13.48 yrs	28.23 ±14.42 yrs
Sex: Male	228 (43.85%)	338 (45.07%)
Female	292 (56.15%)	412 (54.93%)
Onset age (mean age ± SD)	21.25 ± 12.53 yrs	NA
Duration of disease (mean ± SD)	7.8 ± 6.9 yrs	NA
Type of vitiligo		
Generalized	364 (70.00)	NA
Localized	156 (30.00)	NA
Active vitiligo	385 (74.04)	NA
Stable vitiligo	135 (25.96)	NA
Family history	69 (13.27%)	NA

3.2.3 Genotyping of *SOD2* Leu84Phe (C/T) and Thr58Ile (C/T) polymorphisms:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype Leu84Phe (C/T) and Thr58Ile (C/T) polymorphisms of *SOD2* gene (Figure 1A, B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and

reverse primers (Eurofins, Bangalore, India), and 0.3 μL (5U/μL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes *Ear*I and *Eco*RV (New England Biolabs, Beverly, MA) were used for digesting amplicons of Leu84Phe (C/T) and Thr58Ile(C/T) polymorphisms of *SOD2* gene (Table 2) respectively. 5 μL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 μL as per the manufacturer's instruction. The digested products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.5% agarose or 20% polyacrylamide gels stained with ethidium bromide and visualized under UV transilluminator. 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).

3.2.4 Genotyping of *SOD2* Val16Ala (T/C) and Ile82Thr (T/C) polymorphisms:

The genotyping of Val16Ala (T/C) and Ile82Thr (T/C) SNPs of *SOD2* was carried out by dual color hydrolysis probes (FAM and VIC) using LightCycler® 480 Real-Time PCR protocol with background corrected end point fluoroscence analysis using TaqMan SNP genotyping assay (Assay IDs: C_8709053_10 and C_32382851_10 respectively; Life Technologies Corp., California, USA). Real-time PCR was performed in 10 μL volume using LightCycler® 480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. A no-template control (NTC) was used with the SNP genotyping assay.

Table 2. Primers and restriction enzymes used for *SOD2* Leu84Phe (C/T; rs11575993) and Thr58Ile(C/T; rs35289490) SNPs genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs11575993)				*Ear*I
SOD2 C/T F	AGATCCCTGAGCCTAGCAG	54	280	(240 & 40
SOD2 C/T R	CAATCGATTCCTACTGTGCAC			bp)
(rs35289490)				*Eco*RV
SOD2 C/T F	CAGTCTGTATGTTGAGCCATAC	58	195	(177 & 18
SOD2 C/T R	CAGTGCAGGCTGAAGAGAT			bp)
SOD2 expression F	GTTGGCCAAGGGAGATGTTACAG	65	138	-
SOD2 expression R	CAACTCCCCTTTGGGTTCTCCAC			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expression R	CAAATGAGCCCCAGCCTTCT			

3.2.5 Estimation of Superoxide dismutase 2 activity:

The estimation of SOD2 activity in WBC lysate was carried out by the method of Marklund and Marklund (1974) with slight modifications utilizing the inhibition of auto-oxidation of pyrogallol by SOD2 enzyme. The final assay mixture contained 3 ml of Tris buffer (200 mM, pH 8.2) containing air equilibrated 0.2 mM pyrogallol (Merck, India), 1 mM EDTA and 2ul of 1:10 diluted WBC lysate as an enzyme source. 4mM of cyanide was used to inhibit SOD1 and SOD3 activity. The concentration of protein in the WBC lysate was estimated by Lowry's method (Lowry *et al.*, 1951).

Principle:

The autooxidation of pyrogallol, under alkaline condition generates free oxygen radicals which are used by SOD2 present in WBC lysate. Decrease in autooxidation shows indirect evidence of SOD2 activity.

Reagents:

1. Tris buffer 100mM (pH 8.2)

2. Pyrogallol (0.2 mM) dissolved in 0.5 N HCl

3. 4mM Cyanide

Protocol:

The 1:10 dilution of WBC lysate was prepared and O.D. was adjusted to 0.08-0.12 by auto oxidation of pyrogallol at 420 nm and the assay system was followed as given below:

Reagents	**Blank**	**Control**	**Test**
Buffer	1.500 ml	1.500 ml	1.500 ml
DDW	1.350 ml	1.348 ml	1.348 ml
Pyrogallol	0.15 ml	0.15 ml	0.15 ml
WBC lysate (1:10)	-	2 ul	2 ul
Total volume	3 ml	3 ml	3 ml

In all tubes, the reaction was started by the addition of pyrogallol (0.2 mM) and the change in optical density was recorded for 3 mins at an interval of 5 sec at 420 nm. The initial 10 sec were considered as the induction period of the enzyme. The % inhibition of the test system was calculated from the standard graph generated from the auto-oxidation of pyrogallol. The SOD2 specific activity was expressed in units/mg of WBC lyate protein.

Unit: One unit of SOD2 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation.

Specific activity: Units of enzyme/ mg of protein

Folin Lowry Method For Protein Estimation: The method involves both the Biuret reaction, where the peptide bonds of proteins react with Cu^{2+}under alkaline conditions producing Cu^{+}, which reacts with the Folin reagent and the Folin-Ciocalteau reaction which is poorly understood but essentially involves the reduction of phosphomolybdotungstate to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic amino acids. The resultant strong blue color is therefore partly dependent on the tyrosine and tryptophan content of the protein sample.

Reagents:

BSA	1mg /ml
Solution A	10% Na_2CO_3, 2%NaOH, 0.1%NaK tartarate
Solution B	0.5%$CuS0_4.5H_20$
Solution C (freshly prepared): 10ml soln A+40 ml D/W+1 ml soln B	
FC Reagent: Folin Ciocalteau reagent (1:1 dilution)	

Briefly, 2.5 ml of solution C was added to 0.05 ml of WBC lysate, mixed well and incubated for 20 min. Then, 0.25 ml of FC Reagent was added, mixed well and incubated for 45 minutes in dark followed and absorbance was measured at 660nm.

3.2.6 Determination of *SOD2* and *GAPDH* mRNA expression:

3.2.6.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure™- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

3.2.6.2 Real-time PCR:

The expression of *SOD2* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µL volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 15 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data

collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 3D). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

3.2.7 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all the four polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of *SOD2* polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.0125 were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated.

Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi *et al.*, 2005). The linkage disequilibrium (LD) coefficients D' = D/Dmax and r2-values for the pair of the most common alleles at each site were estimated using the Haploview programe version 4.1 (Barrett *et al.*, 2005).

SOD2 specific activity and relative expression of *SOD2* gene in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). 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 (Faul *et al.*, 2007).

3.3 RESULTS

3.3.1 Analysis of association between *SOD2* C/T; Leu84Phe polymorphism and susceptibility to vitiligo:

PCR-RFLP for *SOD2* C/T Leu84Phe polymorphism yielded a 280 bp undigested product corresponding to T allele, and 240 bp and 40 bp digested products corresponding to C allele. The three genotypes identified by 2.5 % agarose gel electrophoresis were: CC homozygous, CT heterozygous and TT homozygous for Leu84Phe polymorphism of *SOD2* gene (Figure 1A).

The genotype and allele frequencies of the C/T Leu84Phe polymorphism in 520 vitiligo patients and 750 controls are summarized in Table 3. The Leu84Phe polymorphism of *SOD2* gene was found to be in significant association with vitiligo patients ($p<0.0001$) when genotypes were compared with chi-squared test-3x2 contingency table (Table 3). The minor allele (T) of Leu84Phe polymorphism was more frequent in the vitiligo group compared to the control group (25.0% versus 11.0%, $p<0.0001$) (Table 3). Both patient and control populations were found to be in Hardy-Weinberg equilibrium for this polymorphism ($p=0.481$ and $p=0.066$ respectively) (Table 3).

Moreover, both generalized and localized vitiligo groups showed significant association of Leu84Phe polymorphism when the genotypes were compared with those of control group ($p<0.0001$) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized and localized vitiligo groups when the alleles were compared with those of control group ($p<0.0001$) (Table 4). The distribution of *SOD2* Leu84Phe genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups ($p>0.05$). Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the Leu84Phe 'T' allele occurred prevalently in the group of patients with active vitiligo compared to the control group (27.00% versus 11.00%, $p<0.0001$) (Table 5). Also, there was statistically significant difference in the distribution of the Leu84Phe 'T' allele between patients with stable vitiligo and control group (19.00% versus 11.00%, $p=0.001$) (Table 5). Interestingly, the Leu84Phe 'T' allele showed significant difference between patients with active vitiligo as compared to stable

vitiligo (27.00% versus 19.00%, p=0.009) (Table 5). This study has 88.76% statistical power for the effect size 0.8 to detect association of Leu84Phe polymorphism of *SOD2* at p<0.05 in patients and control population.

3.3.2 Analysis of association between *SOD2* C/T; Thr58Ile polymorphism and susceptibility to vitiligo:

PCR-RFLP for *SOD2* C/T (Thr58Ile) polymorphism yielded a 195 bp undigested product corresponding to 'C' allele, 177 bp and 18 bp digested products corresponding to 'T' allele. The three genotypes identified on 15 % polyacrylamide gel electrophoresis were: CC homozygous, CT heterozygous and TT homozygous for C/T (Thr58Ile) polymorphism of *SOD2* gene (Figure 1B).

The genotype and allele frequencies of the C/T (Thr58Ile) polymorphism in 520 vitiligo patients and 750 controls are summarized in Table 3. The C/T (Thr58Ile) polymorphism was found to be in significant association with vitiligo patients (p<0.0001) when genotypes were compared with chi-squared test-3x2 contingency table (Table 3). The minor allele (T) of Thr58Ile polymorphism was more frequent in the vitiligo group compared to the control group (41.0% versus 31.0%, p<0.0001) (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.079 and p=0.068 respectively) (Table 3).

Moreover, both generalized and localized vitiligo groups showed significant association of Thr58Ile polymorphism when the genotypes were compared with those of control group (p<0.0001) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized and localized vitiligo groups when the alleles were compared with those of control group (p<0.0001) (Table 4). The distribution of *SOD2* Thr58Ile genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the Thr58Ile 'T' allele occurred prevalently in the group of patients with active vitiligo compared to the control group (46.00% versus 31.00%, p<0.0001) (Table 5). However, there was no significant difference in the distribution of Thr58Ile 'T' allele between patients with stable vitiligo and control group (p=0.132) (Table 5).

Interestingly, the Thr58Ile 'T' allele showed significant difference between patients with active vitiligo as compared to stable vitiligo (46.00% versus 27.00%, $p<0.0001$) suggesting its role in progression of the disease (Table 5). This study has 88.76% statistical power for the effect size 0.8 to detect association of Thr58Ile polymorphism of *SOD2* at $p<0.05$ in patients and control population.

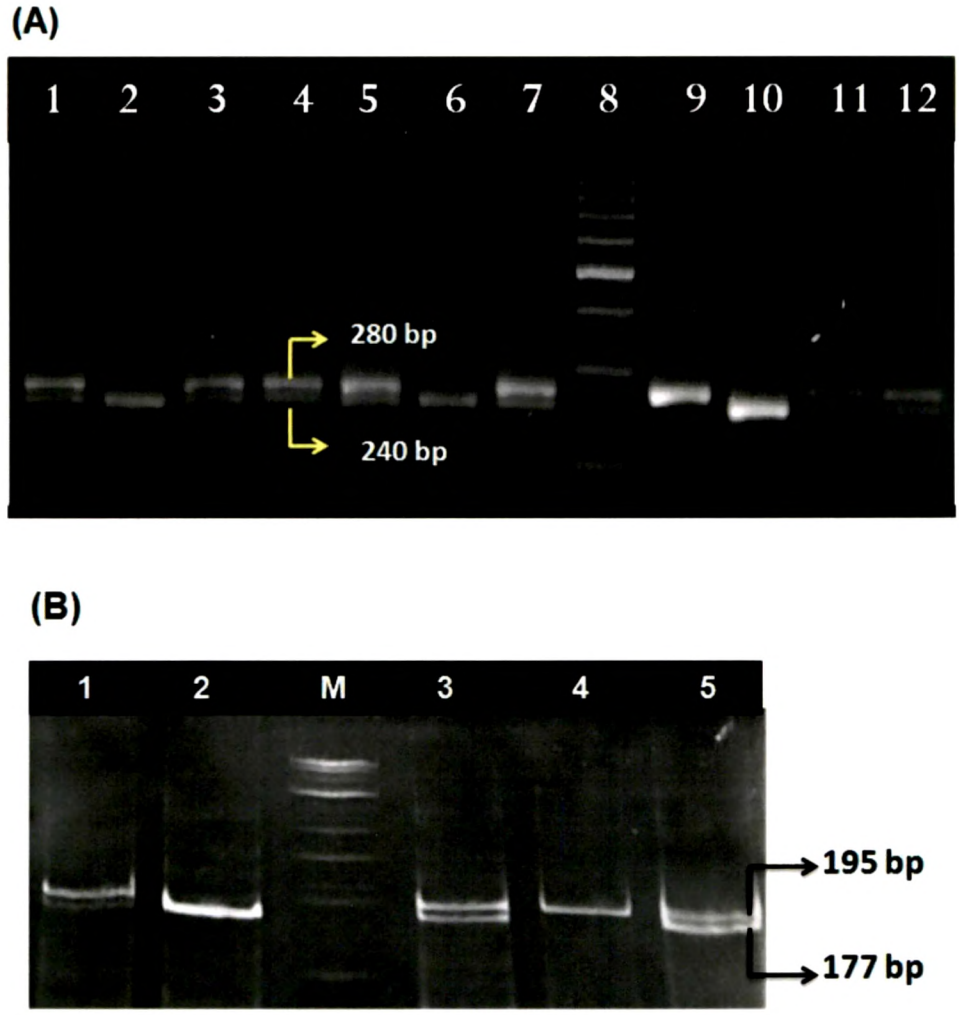


Figure 1. PCR-RFLP analysis of *SOD2* C/T (Leu84Phe) and C/T (Thr58Ile) polymorphisms:

(A) PCR-RFLP analysis of *SOD2* C/T (Leu84Phe) polymorphism on 2.5% agarose gel electrophoresis: lanes: 1, 3, 4, 5, 7, 11 & 12 show heterozygous (CT) genotypes; lane: 2, 6 & 10 show homozygous (CC) genotype; lane: 9 shows homozygous (TT) genotype; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *SOD2* C/T (Thr58Ile) polymorphism on 20% polyacrylamide gel electrophoresis: lanes: 1, 3, & 5 show heterozygous (CT) genotypes; lane: 2 shows homozygous (TT) genotype; lane: 4 shows homozygous (CC) genotype; lane M shows 100 bp DNA ladder.

3.3.3 Analysis of association between *SOD2* T/C; Val16Ala polymorphism and susceptibility to vitiligo:

Real- time PCR based TaqMan SNP genotyping method was carried out by dual color hydrolysis probes labbled with FAM (for 'T' allele) and VIC (for 'C' allele) fluorophores for *SOD2* T/C (Val16Ala) polymorphism which yielded the three genotypes (TT homozygous, TC heterozygous and CC homozygous) as identified by scattered plot using background corrected end point fluoroscence analysis (Figure 2A).

The genotype and allele frequencies of the T/C (Val16Ala) polymorphism in 520 vitiligo patients and 750 controls are summarized in Table 3. The T/C (Val16Ala) polymorphism was not found to be in significant association with vitiligo patients as suggested by the genotype and allele frequencies for the polymorphism (p=0.491; p=0.322) (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.354 and p=0.973 respectively) (Table 3). Also, both generalized and localized vitiligo groups did not show significant association of T/C (Val16Ala) polymorphism when the genotypes were compared with those of control group (p=0.569; p=0.745 respectively) (Table 4). The distribution of *SOD2* T/C (Val16Ala) genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the Val16Ala 'C' allele occurred prevalently in the group of patients with active vitiligo compared to the control group (51.00% versus 41.00%, p=0.006) (Table 5). However, there was no statistically significant difference in the distribution of the Val16Ala 'C' allele between both the groups of patients with active and stable vitiligo and control group (p=0.040; p=0.112) (Table 5). This study has 88.76% statistical power for the effect size 0.08 to detect the association of *SOD2* T/C

(Val16Ala) polymorphism at $p<0.05$ in generalized vitiligo patients and control population.

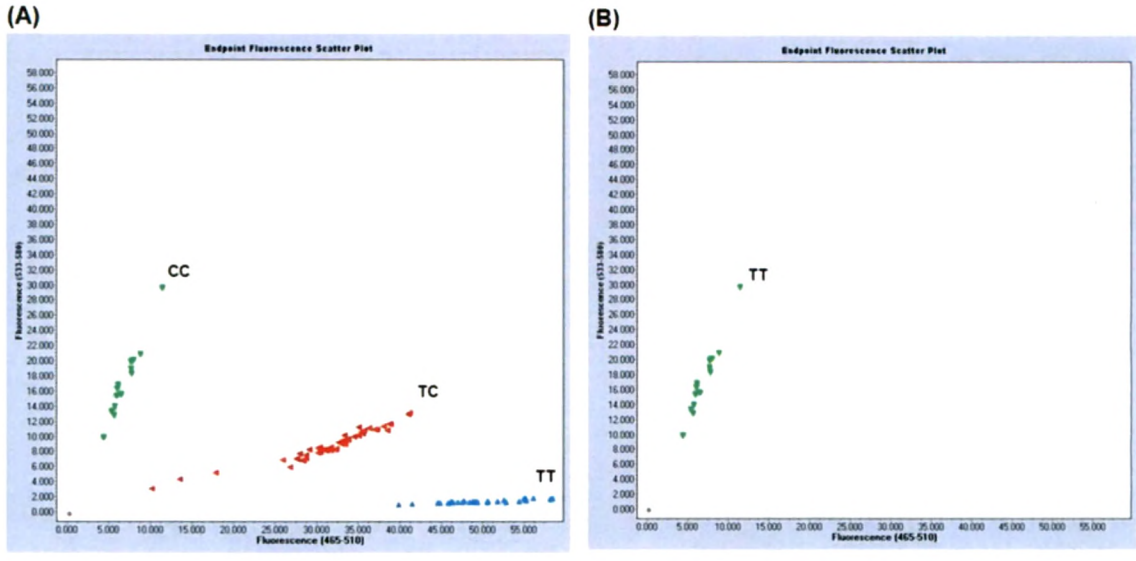


Figure 2. (A) TaqMan end point fluoroscence analysis for *SOD2* Val16Ala (T/C) using dual color hydrolysis probes (FAM and VIC) by LightCycler® 480Real-Time PCR protocol. The three genotypes identified as: TT, TC and CC, based on fluorescence with Channel 465-510 (FAM for 'T' allele) and Channel 536-580 (VIC for 'C' allele).

(B) TaqMan end point fluoroscence analysis for *SOD2* Ile82Thr (T/C) using dual color hydrolysis probes (FAM and VIC) by LightCycler® 480Real-Time PCR protocol. Only single genotype was identified as: TT based on fluorescence with Channel 536-580 (VIC for 'T' allele). There was no fluorescence with Channel 465-510 (FAM for 'C' allele). A no-template control (NTC) was used with each SNP genotyping assay (shown as grey spot).

3.3.4 Analysis of association between *SOD2* T/C; Ile82Thr polymorphism and susceptibility to vitiligo:

Real time PCR based TaqMan SNP genotyping method was carried out by dual color hydrolysis probes labbled with FAM (for 'C' allele) and VIC (for 'T' allele) fluorophores for *SOD2* T/C (Ile82Thr) polymorphism which yield the three genotypes

(TT homozygous, TC heterozygous and CC homozygous) however, in the present study only single genotype was identified by scattered plot using background corrected end point fluoroscence analysis (Figure 2B).

The *SOD2* Ile82Thr (T/C) polymorphism was not found to be associated with vitiligo susceptibility (p=1.00) (Table 3). Interestingly, the polymorphism was found to be monogenic; being only TT alleles presented in control and both the patient groups and hence further analysis was not performed for this polymorphism (Table 4). Also, the distribution of genotype frequencies for *SOD2* Ile82Thr (T/C) polymorphism was not consistent with Hardy-Weinberg expectations in both patient and control groups (p<0.0001).

Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the three polymorphisms [C/T (Leu84Phe), C/T (Ile58Thr) and T/C (Val16Ala)] investigated in the *SOD2* gene were in low LD association [**C/T: C/T** (D' =0.085, r^2=0.002); **C/T: T/C** (D'= 0.081, r^2=0.001); **C/T: T/C** (D'= 0.073, r^2=0.003)]. A haplotype evaluation of the three polymorphic sites was performed and the estimated frequencies of the haplotypes differed significantly between vitiligo patients and controls (global p-value=6.33e-032) (Table 6). Intersetingly, the frequency of susceptible haplotype 'TTC' containing the minor alleles of all the three polymorphisms were significantly higher in vitiligo patients as compared to controls (5.1% vs 1.1%; p=1.98e-008) and increased the risk of vitiligo by 4.6-fold [odds ratio (OR): 4.696; 95% confidence interval (CI): (2.601~8.477)] (Table 6). Also three other haplotypes with one or two susceptible alleles: 'TTT' and 'TCT' and 'TCC' were significantly increased in vitiligo patients as compared to controls (p=2.78e-006, p=1.13e-014; p=3.97e-015). However, the wild type haplotype 'CCT' was more frequently observed in control group as compared to the patient group (30.1% vs 21.5%; p=1.04e-005) (Table 6).

Table 3. Association studies for *SOD2* gene, C/T (Leu84Phe), C/T (Thr58Ile), T/C (Val16Ala) and T/C (Ile82Thr) polymorphisms in vitiligo patients from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 520)	(n = 750)			
	CC	297 (0.57)	599 (0.80)		0.481	
	CT	188 (0.36)	137 (0.18)	<0.0001	(P)	
rs11575993	TT	35 (0.07)	14 (0.02)			
(C/T;	Allele				0.066	0.3746
Leu84Phe)	C	782 (0.75)	1335(0.89)	<0.0001	(C)	(0.3023-
	T	258 (0.25)	165 (0.11)			0.4642)
	Genotype	(n = 520)	(n = 750)			
	CC	189 (0.36)	364 (0.49)		0.068	
	CT	232 (0.45)	302 (0.40)	<0.0001	(P)	
rs35289490	TT	99 (0.19)	84 (0.11)			
(C/T;	Allele				0.079	0.6473
Thr58Ile)	C	610 (0.59)	1030 (0.69)	<0.0001	(C)	(0.5490-
	T	430 (0.41)	470 (0.31)			0.7633)
	Genotype	(n = 520)	(n = 750)			
	TT	140 (0.27)	225 (0.30)		0.973	
rs4880	TC	260 (0.50)	360 (0.48)	0.491	(P)	
(T/C;	CC	120 (0.23)	165 (0.22)			
Val16Ala)	Allele				0.354	0.9200
	T	540 (0.52)	810 (0.54)	0.322	(C)	(0.7852-
	C	500 (0.48)	690 (0.46)			1.078)
	Genotype	(n=520)	(n = 750)			
	TT	520 (1.00)	750 (1.00)		<0.0001	
rs1141718	TC	0 (0.00)	0 (0.00)	1.000	(P)	
(T/C;	CC	0 (0.00)	0 (0.00)			
Ile82Thr)	Allele				<0.0001	
	T	1040(1.0)	1500 (1.00)	1.000	(C)	
	C	0 (0.00)	0 (0.00)			

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.0125$ due to Bonferroni's correction.

Table 4. Association studies for SOD2 gene, C/T (Leu84Phe), C/T (Thr58Ile) and T/C (Val16Ala) polymorphisms in generalized and localized vitiligo patients from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo (Freq.)	Localized Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 364)	(n = 156)	(n = 750)		0.875	
	CC	196(0.54)	101 (0.65)	599(0.80)	<0.0001[a]	(GV)	2.919[c]
	CT	143(0.39)	45 (0.29)	137(0.18)	<0.0001[b]		(2.317-
rs11575993	TT	25(0.07)	10 (0.06)	14 (0.02)		0.117	3.677)
(C/T;	Allele					(LV)	
Leu84Phe)	C	535(0.73)	247 (0.79)	1335(0.89)	<0.0001[c]		2.129[d]
	T	193(0.27)	65 (0.21)	165(0.11)	<0.0001[d]	0.066	(1.550-
						(C)	2.925)
	Genotype	(n = 364)	(n = 156)	(n = 750)		0.433	
	TT	105(0.29)	84 (0.54)	364(0.49)	<0.0001[a]	(GV)	0.4248[c]
	TC	174(0.48)	58 (0.37)	302(0.40)	<0.0001[b]		(0.3185-
rs35289490	CC	85 (0.23)	14 (0.09)	84 (0.11)		0.389	0.5665)
(C/T;	Allele					(LV)	
Thr58Ile)	T	384(0.53)	226 (0.72)	1030(0.69)	<0.0001[c]		0.5094[d]
	C	344(0.47)	86 (0.28)	470 (0.31)	<0.0001[d]	0.079	(0.4247-
						(C)	0.6110)
	Genotype	(n= 364)	(n = 156)	(n = 750)		0.978	
	TT	98 (0.27)	42 (0.27)	225(0.30)	0.569[a]	(GV)	0.9200[c]
rs4880	TC	182(0.50)	78 (0.50)	360 (0.48)	0.745[b]		(0.7705-
(T/C;	CC	84 (0.23)	36 (0.23)	165 (0.22)		0.985	1.099)
Val16Ala)	Allele					(LV)	
	T	378(0.52)	162 (0.52)	810 (0.54)	0.381[c]		0.9200[d]
	C	350(0.48)	150 (0.48)	690 (0.46)	0.533[d]	0.354	(0.7206-
						(C)	1.175)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(GV) refers to Generalized Vitiligo, (LV) Localized Vitiligo and (C) refers to Controls,

[a] Generalized Vitiligo vs. Controls using chi-squared test with 3 × 2 contingency table,

[b]Localized Vitiligo vs. Controls using chi-squared test with 3 × 2 contingency table,

[c] Generalized Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table,

[d] Localized Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.0125$ due to Bonferroni's correction.

Table 5. Association studies for *SOD2* gene, C/T (Leu84Phe), C/T (Thr58Ile) and T/C (Val16Ala) polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 385)	(n = 135)	(n = 750)		0.831	0.6334[a]
	CC	205 (0.57)	92 (0.68)	599 (0.80)	0.009[a]	(AV)	(0.4490-
	CT	153 (0.40)	35 (0.26)	137 (0.18)	<0.0001[b]		0.8934)
rs11575993	TT	27 (0.07)	08 (0.06)	14 (0.02)	0.002[c]	0.074	
(C/T;	Allele					(SV)	0.3362[b]
Leu84Phe)	C	563 (0.73)	219(0.81)	1335(0.89)	0.009[a]		(0.2679-
	T	207 (0.27)	51 (0.19)	165 (0.11)	<0.0001[b]	0.066	0.4219)
					0.001[c]	(C)	
							0.5307[c]
							(0.3758-
							0.7495)
	Genotype	(n = 385)	(n = 135)	(n = 750)		0.439	0.4185[a]
	TT	114 (0.30)	75 (0.56)	364 (0.49)	<0.0001[a]	(AV)	(0.3086-
	TC	184 (0.48)	48 (0.36)	302 (0.40)	<0.0001[b]		0.5676)
rs35289490	CC	87 (0.22)	12 (0.08)	84 (0.11)	0.301[c]	0.291	
(C/T;	Allele					(SV)	0.5251[b]
Thr58Ile)	T	412 (0.54)	198(0.73)	1030(0.69)	<0.0001[a]		(0.4392-
	C	358 (0.46)	72 (0.27)	470 (0.31)	<0.0001[b]	0.079	0.6280)
					0.132[c]	(C)	
							1.255[c]
							(0.9380-
							1.679)
	Genotype	(n= 385)	(n = 135)	(n = 750)		0.573	0.6699[a]
	TT	91 (0.24)	49 (0.36)	225 (0.30)	0.012[a]	(AV)	(0.5058-
rs4880	TC	198 (0.51)	62 (0.46)	360 (0.48)	0.072[b]		0.8872)
(T/C;	CC	96 (0.25)	24 (0.18)	165 (0.22)	0.283[c]	0.570	
Val16Ala)	Allele					(SV)	0.8300[b]
	T	380 (0.49)	160(0.59)	810 (0.54)	0.006[a]		(0.6974-
	C	390 (0.51)	110(0.41)	690 (0.46)	0.040[b]	0.354	0.9878)
					0.112[c]	(C)	
							1.239[c]
							(0.9523-
							1.612)

'n' represents number of Patients/ Controls

HWE refers to Hardy-Weinberg Equilibrium,

AV refers to Active Vitiligo, SV refers to Stable Vitiligo and C refers to controls,

[a]Active Vitiligo vs. Stable Vitiligo,

[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Values are significant at $p \leq 0.0125$ due to Bonferroni's correction.

Table 6. Distribution of haplotypes frequencies for *SOD2* gene, C/T (Leu84Phe), C/T (Ile58Thr) and T/C (Val16Ala) polymorphisms among generalized vitiligo patients and controls.

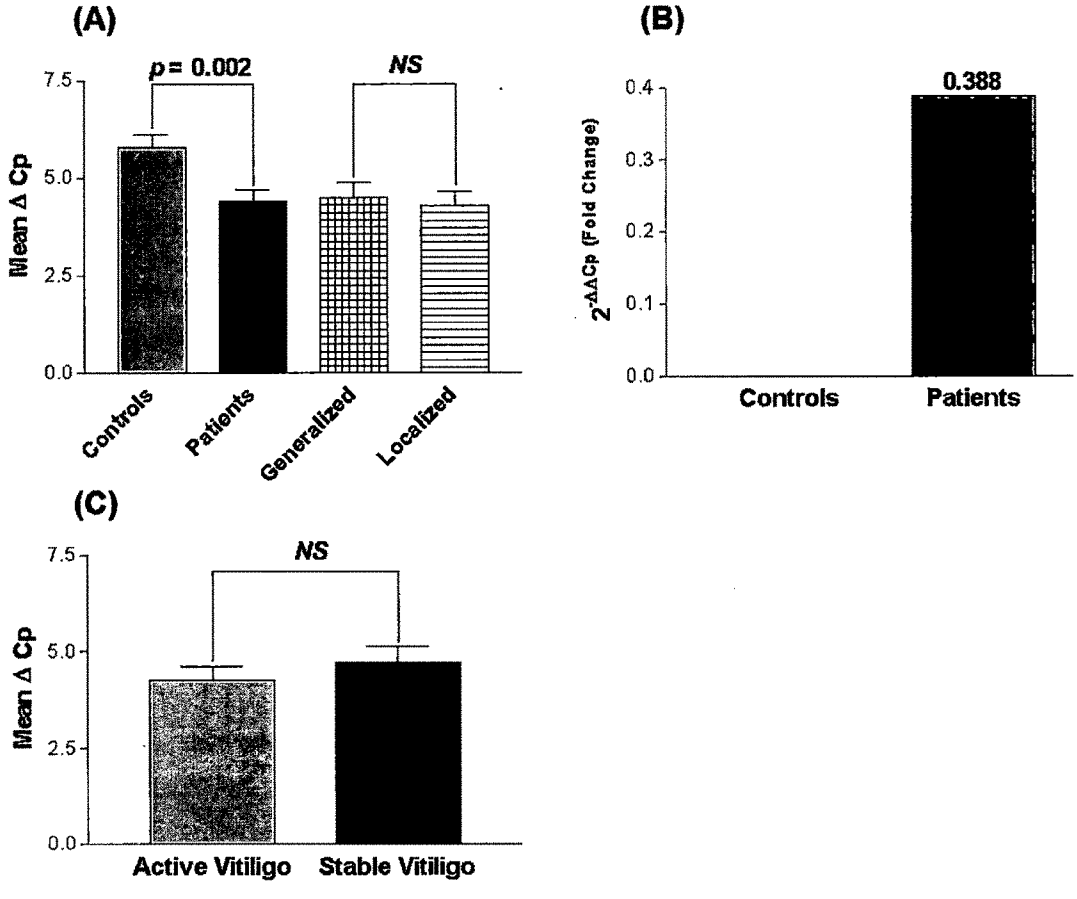
Haplotype (C/T (Leu84Phe), C/T (Ile58Thr) and T/C (Val16Ala)	Generalized Vitiligo Patients (Freq. %) (n=926)	Controls (Freq. %) (n=1358)	*p* for Association	p_{global}	Odds ratio (95% CI)
C C C	171.30(0.207)	390.25(0.287)	3.12e-005		0.647 [0.527~0.795]
C C T	177.61(0.215)	408.62(0.301)	1.04e-005		0.635 [0.519~0.778]
C T C	117.72(0.143)	198.39(0.146)	0.807022	6.33e-032	0.970 [0.758~1.241]
C T T	145.37(0.176)	286.73(0.211)	0.043846		0.797 [0.638~0.994]
T C C	68.81(0.083)	19.99(0.015)	3.97e-015		6.073 [3.662~10.072]
T C T	62.28(0.075)	16.14(0.012)	1.13e-014		6.772 [3.889~11.792]
T T C	42.17(0.051)	15.36(0.011)	1.98e-008		4.696 [2.601~8.477]
T T T	40.73(0.049)	20.51(0.015)	2.78e-006		3.377 [1.972~5.784]

CI represents Confidence Interval,

(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

3.3.5 Relative gene expression of *SOD2* in patients with vitiligo and controls:

Our *SOD2* gene expression studies showed significant increase in expression of *SOD2* transcripts in 166 vitiligo patients compared to 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.002) (Figure 3A). However, there was no significant difference in expression of *SOD2* between generalized and localized vitiligo patients (p=0.726) (Figure 3A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.388 fold change in the expression of *SOD2* transcript in patients as compared to controls (Figure 3B). In addition, we also checked the effect of *SOD2* expression on progression of the disease i.e. active and stable cases of vitiligo (Figure 3C); however, no significant difference was observed between active and stable vitiligo patients (p=0.481).



(D)

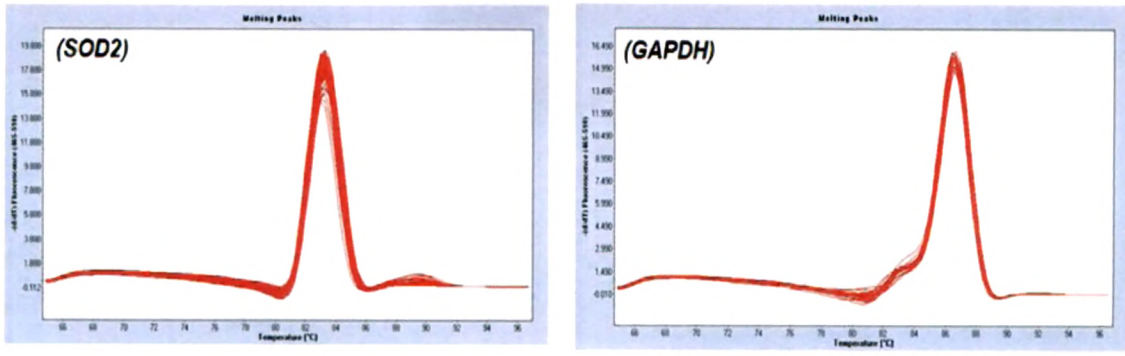


Figure 3. Relative gene expression of *SOD2* in controls and vitiligo patients:

(A) Expression of *SOD2* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *SOD2* as compared to controls (Mean ΔCp ± SEM: 4.421 ± 0.2848 vs 5.788 ± 0.3236; p=0.002). Generalized vitiligo patients did not show difference in mRNA levels of *SOD2* as compared to localized patients (Mean ΔCp ± SEM: 4.499 ± 0.4037 vs 4.291 ± 0.3606; p=0.726) [*NS* = non-significant].

(B) Expression fold change of *SOD2* transcripts in 166 vitiligo patients against 175 controls showed 0.388 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *SOD2* transcripts with respect to activity of the disease in 121 patients with active vitiligo and 45 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients did not show difference in mRNA levels of *SOD2* as compared to stable vitiligo patients (Mean ΔCp ± SEM: 4.254 ± 0.3644 vs 4.700 ± 0.4365; p=0.481).

(D) Melt curve analysis of *SOD2* and *GAPDH* showing specific amplification.

3.3.6 Estimation of SOD2 activity in vitiligo patients and controls:

We have also analyzed the SOD2 specific activity in WBC-lysate from 520 vitiligo patients and 750 controls. SOD2 activity differed significantly between vitiligo patients and controls (p<0.0001) (Figure 4A). In particular, vitiligo patients showed 2.3 fold higher SOD2 activity as compared to controls. However, there was no significant difference in SOD2 activity between generalized and localized vitiligo patients (p=0.406) (Figure 4A).

Analysis based on the stage of progression of vitiligo revealed that active vitiligo patients had significant increase in SOD2 activity as compared to the patients with stable vitiligo (p=0.011) which suggests the involvement of SOD2 in disease progression (Figure 4B).

3.3.7 Correlation of *SOD2* C/T (Leu84Phe), C/T (Thr58Ile) and T/C (Val16Ala) genotypes with SOD2 activity in vitiligo patients:

Further, the SOD2 activity was correlated with the genotypes obtained for Leu84Phe, Thr58Ile and Val16Ala polymorphisms (Figure 4). Interestingly, SOD2 activity was significantly increased in patients with susceptible Thr58Ile TT genotypes and heterozygous CT genotypes as compared to controls (p=0.021; p=0.016) suggesting the involvement of Thr58Ile'T' allele in increased activity of SOD2.

No significant difference was observed in SOD2 activity in patients with CC genotypes as compared to controls (p=0.596) (Figure 4D).

However, SOD2 activity did not differ significantly between patients with Leu84Phe TT, CT and CC genotypes as compared to controls (p=0.854; p=0.712; p=0.817) (Figure 4C). Moreover, there was no significant difference in SOD2 activity in patients with Val16Ala TT, TC and CC genotypes as compared to controls (p=0.866; p=0.202; p=0.071) (Figure 4E) suggesting that Leu84Phe and Val16Ala polymorphisms are not involved in increased SOD2 activity in patients.

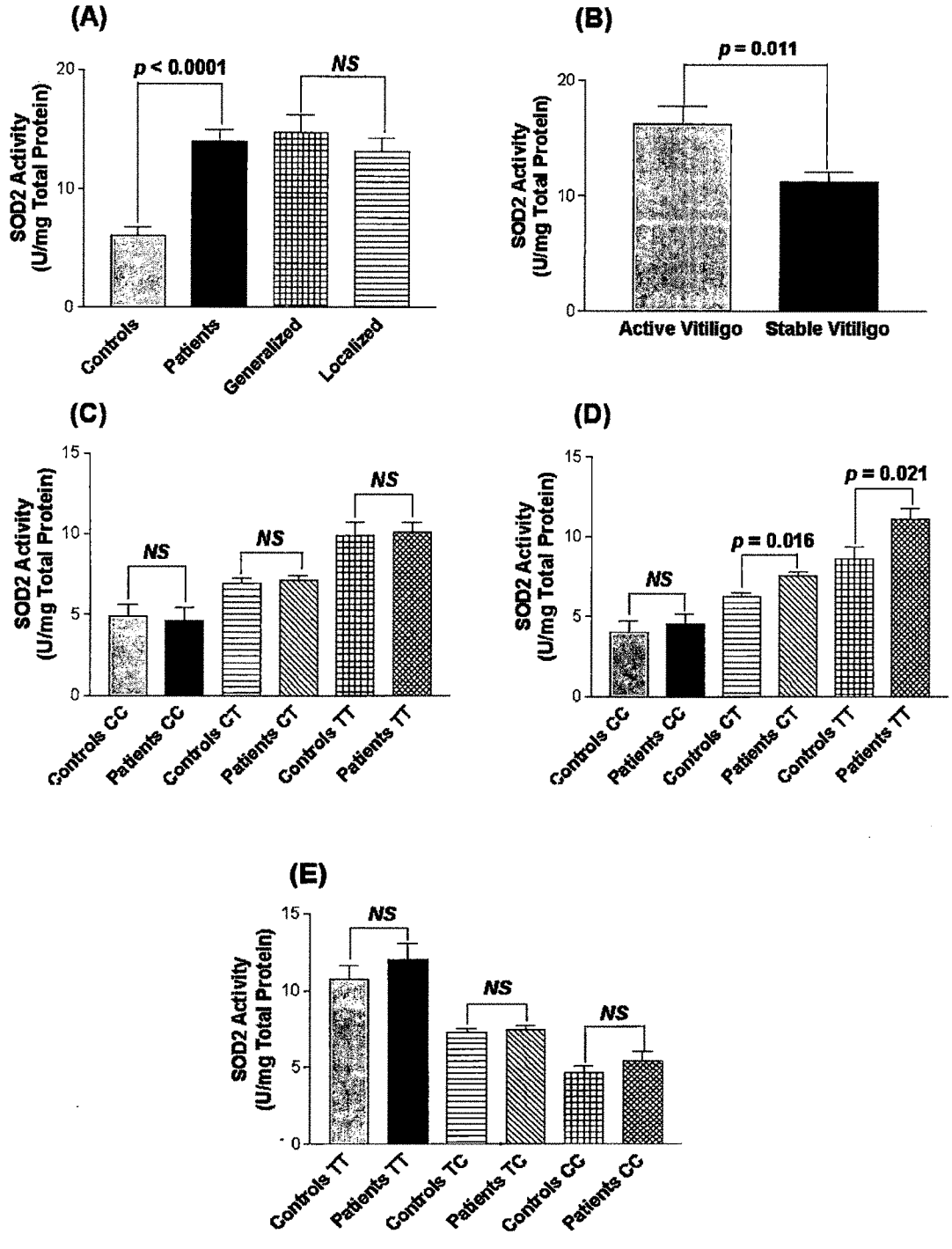


Figure 4. SOD2 specific activity in controls and vitiligo patients:

(A) SOD2 specific activity in 520 vitiligo patients and 750 controls. Vitiligo patients showed significantly increased SOD2 activity as compared to controls (Mean ± SEM: 13.98 ± 0.9810 vs 6.108 ± 0.6305; $p<0.0001$). Generalized vitiligo patients (n=364) did not show difference in SOD2 activity as compared to localized patients (n=156) (Mean ± SEM: 14.73 ± 1.488 vs 13.08 ± 1.211; $p=0.406$) [*NS* = non-significant].

(B) SOD2 specific activity with respect to activity of the disease in 385 patients with active vitiligo and 135 patients with stable vitiligo. Active vitiligo patients showed significant difference in SOD2 activity as compared to stable vitiligo patients (Mean ± SEM: 16.25 ± 1.601 vs 11.25 ± 0.8688; p=0.011).

(C) SOD2 specific activity with respect to *SOD2* C/T (Leu84Phe) polymorphism in 520 vitiligo patients and 750 controls. No significant difference was observed in SOD2 activity with TT (Mean ± SEM: 10.12 ± 0.6632 vs 9.921 ± 0.8360; p=0.854), CT (Mean ± SEM: 7.106 ± 0.3319 vs 6.924 ± 0.3395; p=0.712) and CC (Mean ± SEM: 4.606 ± 0.8761 vs 4.883 ± 0.7670; p=0.817) genotypes between vitiligo patients and controls.

(D) SOD2 specific activity with respect to *SOD2* C/T (Thr58Ile) polymorphism in 520 vitiligo patients and 750 controls. Vitiligo patients showed significant increase in SOD2 activity with TT (Mean ± SEM: 11.14 ± 0.6645 vs 8.601 ± 0.7934; p=0.021) and CT (Mean ± SEM: 7.566 ± 0.2746 vs 6.262 ± 0.2439; p=0.016) genotypes as compared to controls. There was no significant difference in the activity of SOD2 with CC genotypes (Mean ± SEM: 4.531 ± 0.6291 vs 4.009 ± 0.6917; p=0.596) as compared to controls.

(E) SOD2 specific activity with respect to *SOD2* T/C (Val16Ala) polymorphism in 520 vitiligo patients and 750 controls. No significant difference was observed in SOD2 activity with CC (Mean ± SEM: 5.448 ± 0.5841 vs 4.683 ± 0.4531; p=0.817), TC (Mean ± SEM: 7.498 ± 0.2637 vs 7.290 ± 0.2752; p=0.595) and TT Mean ± SEM: 12.04 ± 0.9936 vs 10.74 ± 0.8725; p=0.361) genotypes between vitiligo patients and controls. [*NS* = non-significant]

3.4 DISCUSSION

ROS are produced because approximately 2–3% of the oxygen atoms taken up by the mitochondria are reduced insufficiently (Valko *et al.*, 2004). ROS can oxidize and damage nucleic acids, proteins and lipids thereby altering their stability and function (Evans *et al.*, 2004; Chen *et al.*, 2008; Xie *et al.*, 2008). Thus, protein modifications (such as protein carbonylation and nitration) and the formation of lipid peroxidation adducts (e.g. 4-hydroxynonenal) can be the results of ROS damage.

Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction as H_2O_2 accumulation is observed in the epidermis of active vitiligo

patients (Bindoli *et al.,* 1992; Schallreuter *et al.,* 1996). One of the important reasons for increased epidermal H_2O_2 levels is an imbalance in the antioxidant system. Previously, we have reported significant increase in erythrocyte lipid peroxidation levels in patients of vitiligo in all age groups as compared to healthy controls from Gujarat (Agrawal *et al.,* 2004; Shajil and Begum, 2006). The study showed that systemic oxidative stress might precipitate the pathogenesis of vitiligo in susceptible patients (Agrawal *et al.,* 2004). Systemic oxidative stress is also reported to be elevated in patients (Hazneci *et al,* 2005). The mechanism underlying this etiology is not yet known but increased systemic oxidative stress is generated due to altered antioxidant system which affects the epidermal melanocytes leading to vitiligo manifestation.

Mitochondria are a major site of ATP production through oxidative phosphorylation, and they also participate in other cellular functions such as cell cycle regulation, stress response (Manoli *et al.*, 2007), and different aspects of metabolism (Bowsher and Tobin, 2001; Chen *et al.*, 2006; Eaton *et al.*, 1996; Leverve, 2007; Manoli *et al.*, 2007; Newsholme *et al.*, 2007). Mitochondria are also important for apoptosis (Gulbins *et al.*, 2003; Spierings *et al.*, 2005) due to altered mitochondrial function (Green and Reed, 1998) and the presence of many factors that induce apoptosis upon release from mitochondria, including cytochrome c (Scheffler, 1999), apoptosis inducing factor (AIF), endonuclease G, Smac/Diablo, and Omi/Htr2 (Adams, 2003). Manganese superoxide dismutase (SOD2) is an antioxidant enzyme, located in the mitochondrial matrix that appears to have strategic advantage in providing an initial defense against oxidative damage by ROS. This enzyme catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen (Barra *et al.,* 1984). Given that mitochondria are the major sites for cellular metabolism and thus of ROS production, SOD2 is very important in protecting cells from ROS-induced oxidative damage.

A number of studies have identified the association of *SOD2* genetic polymorphisms with various diseases e.g. type II diabetes, hypertension etc. (Nakanishi *et al.*, 2008; Hirooka, 2008; Arsova *et al.*, 2008; Mikhak *et al.*, 2008). The presence of *SOD2* single-nucleotide polymorphisms (SNPs) and the potential effects of these SNPs on human SOD2 have been briefly reviewed previously (St Clair and Kasarskis, 2003).

The present study investigated four non-synonymous SNPs: Leu84Phe (C/T; rs11575993), Thr58Ile(C/T; rs35289490), Val16Ala (T/C; rs4880) and Ile82Thr (T/C; rs1141718) in SOD2 gene, out of which two SNPs: Thr58Ile(C/T; rs35289490), Val16Ala (T/C; rs4880) are well documented in several diseases e.g. Parkinson's disease, diabetes mellitus and malignancies (Shimoda-Matsubayashi *et al.*,1996; Pociot *et al.*,1993; Ambrosone, *et al.*,1999).

The transport of SOD2 into mitochondria is mediated through the interaction of the mitochondrial targeting sequence with receptors on the mitochondrial membrane. The Val16Ala (T/C) polymorphism in the mitochondrial targeting sequence may influence the efficiency of SOD2 transport. Val16Ala is implicated in decreased efficiency of SOD2 transport into target mitochondria in Val allele carriers (Sutton *et al.*, 2003). The 16Ala polymorphism results in the formation of α-helix and the 16Val takes a β-sheet structure and Val allele was predicted to disrupt this structure (Shimoda-Matsubayashi *et al.*, 1996). The α-helix structure is important for the effective transport of precursor proteins into mitochondria (Lemire *et al.*, 1989). The amino acid substitution (Val/Ala) at position 16 of the mitochondrial targeting sequence may lead to misdirected trafficking, followed by the alteration of SOD2 activity in human mitochondria.

Several studies have suggested the association of the SOD2 Val16Ala 'T' allele with an increased risk of breast (Ambrosone *et al.*, 1999), prostate (Woodson *et al.*, 2003) and ovarian cancer (Olson *et al.*, 2004). In addition, the variant 'T'allele was associated with sporadic motor neuron disease (Van-Landeghem *et al.*, 1999), exudative agerelated macular degeneration (Kimura *et al.*, 2000), diabetic nephropathy (Nomiyama *et al.*, 2003) and ankylosing sypondylitis (Yen *et al.*, 2003), which are all related to oxidative stress and abnormal free radical defence mechanisms.

The present study suggests significant association of Val16Ala polymorphism with active vitiligo cases. In particular, the Val16Ala 'T' allele is prevalent in patient group as compared to controls indicating the important role played by 16Ala (T) allele in disease progression (Table 5). However, overall vitiligo patients did not show significant association with this polymorphism (Table 3). It is reported that carriers of 16Ala (T) allele have 30-40% more activity of SOD2 compared to 16Val (C) allele

carriers (Sutton *et al.*, 2003). The genotype-phenotype correlation study for this polymorphism revealed an increase in SOD2 activity with Val16Ala TT genotypes as compared to TC and CC genotypes which implicates the role of Val16Ala polymorphism in increased activity of SOD2; however, the activity was not significant between patients and controls harbouring these genotypes (Figure 4E).

Overall, vitiligo patients exhibited significantly higher (2.3 fold) SOD2 activity as compared to controls suggesting the high oxidative stress in patients (Figure 4A). Moreover, the SOD2 activity was also increased in patients with active vitiligo (1.5 fold) as compared to stable vitiligo, suggesting the important role of SOD2 in disease progression (Figure 4C). The SOD2 gene expression analysis showed significantly higher SOD2 transcripts levels (1 fold) as compared to controls (Figure 3A). However, there was no significant difference in SOD2 expression between active and stable patients suggesting that increased activity in active vitiligo patients may be mainly contributed by Val16Ala polymorphism (Figure 3C).

A wide variety of compounds induce transcription of *SOD2*. Cytokines such as interleukin (IL)-1 (Masuda *et al.*, 1988; Visner *et al.*, 1992; Dougall and Nick, 1991), IL-4, IL-6 (Dougall and Nick, 1991), TNF-α (Visner *et al.*, 1992; Wong and Goeddel, 1988), lipopolysaccharide (LPS) (Visner *et al.*, 1990), and IFN-γ (Harris *et al.*, 1991) are potent activators of SOD2 in different tissues and cell types. The cytokine inducible enhancer regions in SOD2 contain binding sites for NF-kB, C/EBP, and NF-1 transcription factors. Our recent studies also suggest increased levels of cytokines such as TNF-α, TNF-β, IFN-γ (unpublished data) and IL-4 (Imran *et al.*, 2012) in vitiligo patients which might be responsible for the induction of SOD2 as observed in the present study. The redox-sensitive transcriptional factor NF-κB acts as a regulator of genes by serving as an immediate responder to harmful cellular stimuli like high ROS. NF-κB is identified as the most crucial transcriptional factor regulating *SOD2* induction (Eastgate *et al.*, 1993).

The SOD2 Thr58Ile (C/T) polymorphism is related to the potency of SOD2. The Thr58Ile SNP disrupts the native tetrameric form of SOD2 and promotes a dimeric form in solution (Borgstach *et al.*, 1996). In Thr58Ile polymorphism (C/T), SOD2 with threonine has a weaker antioxidant activity than that with isoleucine (Ho *et al.*,1998). The polymorphism, Thr58Ile, affects the stability of protein tetrameric

interface and reduces the biological activity of SOD2 (Borgstahl *et al.*, 1996; Ho and Crapo, 1988). The 58Ile protein form showed three-fold higher activity than the 58Thr form (Zhang *et al.*, 1999). However, Thr mutant form has increased thermal instability and accelerated thermal inactivation (Borgstahi 1992; Borgstahl *et al.*, 1996) which decreases the activity of SOD2. Intersetingly, we found significant association of Thr58Ile polymorphism with vitiligo being 'T' allele prevalent in vitiligo group (Table 3). This association was consistent with other subtypes of vitiligo i.e. generalized, localized, active and stable vitiligo patients (Table 4 & 5). This suggests the profound effect of 58Ile (T) allele in vitiligo susceptibility. The genotype-phenotype correlation of this SNP revealed significantly higher SOD2 activity in patients harbouring TT and CT genotypes as compared to those of controls (Figure 4D). Previous studies suggested the association of Ile58Thr polymorphism with neurodegenerative diseases involving a decrease in SOD2 levels (Checkoway *et al.*, 1998). No association between the Ile58Thr polymorphism and Parkinson's disease, type 1 diabetes and bladder cancer was found (Grasbon-Frodl *et al.*, 1999; Chistyakov *et al.*, 2001; Paz-y-Min˜o *et al.* (2010). However this mutation is more likely to be involved in hereditary aging-related neurodegenerative disease (Borgstahl *et al.*, 1996).

Furthermore, the present study found significant association of the non-synonymous SNP: Leu84Phe (C/T) with vitiligo. However, Ile82Thr (T/C) showed the presnce of only single allele i.e. 'T' in both control and patient groups (Table 3). There was no signifcant difference in SOD2 activity of patients harbouring the different genotypes of Leu84Phe polymorphism, suggesting that the polymorphism does not play an important role in vitiligo pathogenesis; however the SOD2 activity was increased with subjects harbouring TT and CT genotypes as compared to CC genotypes suggesting the effect of 'T' allele on increased activity of SOD2 (Figure 4C). However, the prevalence of Leu84Phe minor allele 'T' was significantly high in active patients of vitiligo as compared to stable patients suggesting the important role of 'T' allele in progression of the disease (Table 5). There are no reports available for these two non-synonymous SNPs in any disease pathogenesis. Intersetingly, the frequency of susceptible haplotype 'TTC' containing the minor alleles of all the three polymorphisms were significantly higher in vitiligo patients as compared to controls and increased the risk of vitiligo by 4.6-fold (Table 6). Also, three other haplotypes

with one or two susceptible alleles: 'TTT' and 'TCT' and 'TCC' were significantly increased in vitiligo patients as compared to controls suggesting the important role of susceptible alleles in vitiligo.

In conclusion, the present study signifies the important role of SOD2 in oxidative stress mediated pathogenesis and progression of vitiligo. In particular, the association of Thr58Ile had a profound effect on SOD2 activity in patients and the increased activity of SOD2 may in part be contributed by the polymorphism as well as increased SOD2 mRNA levels which in turn results in increased H_2O_2 production. As the downstream antioxidant system in vitiligo patients was found to be disturbed, the H_2O_2 might not be properly removed and may finally lead to oxidative damage to the melanocytes.

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