CHAPTER II

ROLE OF SUPEROXIDE DISMUTASE 1 (SOD1) IN VITILIGO SUSCEPTIBILITY

2.1 INTRODUCTION

Oxidative stress is a major form of assault on the skin. Skin exposure to ionizing and UV radiation and/or xenobiotics/drugs generates ROS in excessive quantity. It overwhelms tissue antioxidants and other oxidant-degrading pathways. Uncontrolled release of ROS and increased lipid peroxidation are seen in multiple skin disorders such as atopic dermatitis, psoriasis, acne vulgaris, pemphigus vulgaris (PV), lichen planus, alopecia areata and vitiligo (Yesilova *et al.*, 2012). Oxidative stress has been implicated as the initial triggering event in vitiligo pathogenesis leading to melanocyte destruction (Schallreuter *et al.*, 1999a; Maresca *et al.*, 1997) which is marked by accumulation of H_2O_2 in the epidermis of vitiligo patients (Schallreuter *et al.*, 2001). Defective recycling of tetrahydrobiopterin in vitiligo epidermis is related with the intracellular production of H_2O_2 (Schallreuter *et al.*, 1994; Schallreuter *et al.*, 1999b). In addition, a compromised antioxidant status with a significant reduction in catalase activity and increase in SOD activity has been demonstrated in both lesional and non-lesional epidermis (Schallreuter *et al.*, 1991; Sravani *et al.*, 2009) as well as in melanocytes (Maresca *et al.*, 1997). Interestingly, antioxidant imbalance has also been observed in the peripheral blood mononuclear cells of active vitiligo patients. This was correlated with an increased intracellular production of reactive oxygen species due to mitochondrial impairment (Dell'Anna *et al.*, 2001), supporting the concept of a possible systemic oxidative stress in vitiligo. Impaired redox status of the epidermal melanin unit acts as 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 and H_2O_2 overproduction leads to catalase inactivation in the epidermal melanocytes (Schallreuter *et al.*, 1996, Maresca *et al.*, 1997).

In normal cellular processes, SOD1 is a major antioxidant enzyme that catalyzes the conversion of superoxide anion to hydrogen peroxide and molecular oxygen. Hydrogen peroxide in turn converted to oxygen and water by the action of catalase and glutathione peroxidase. As a result cell maintains its normal integrity and function. Sometimes equilibrium of antioxidants and oxidants gets imbalanced which leads to accumulation of free radicals resulting into the accumulation of oxidative stress. This accumulated oxidative stress causes DNA damage (Mohamed and Salem,

2009), lipid and protein peroxidation. Many proteins and peptides get altered or even show complete loss of functionality due to H_2O_2-mediated oxidation.

Vitiligo is an acquired hypomelanotic skin disorder, characterized by milky white patches of different size and shape. Though vitiligo is extensively addressed in the past six decades, its etiology is still being debated (Taieb 2000; Le Poole *et al.,* 1993; Ortonne and Bose 1993; Cucchi *et al.,* 2003; Ongenae *et al.,* 2003; Boisseau-Garsuad *et al.,* 2002). Vitiligo susceptibility is a complex genetic trait that may involve genes for melanin synthesis, response to oxidative stress and regulation to autoimmunity. In order to explore the genetic susceptibility, systematic study of each gene governing oxidative stress, melanin synthesis, and regulation of autoimmunity is essential. Several hypotheses were proposed to explain the pathogenesis of vitiligo and oxidative stress hypothesis considers a systemic involvement in the course of the disease (Picardo *et al.,* 1994; Yildirim *et al.,* 2003). Oxidative stress could act as the initial triggering event in melanocyte degeneration.

Superoxide dismutases are metalloenzymes found widely distributed in prokaryotic and eukaryotic cells (Fridovich, 1995). Superoxide dismutases scavenge the superoxide radicals and reduce its toxicity by converting it to H_2O_2 (Schallreuter *et al.,* 1991). Three distinct isoforms of SOD have been identified in mammals. Two isoforms of SOD have Cu and Zn at their catalytic center and are localized to either intracellular cytoplasmic compartments (CuZn or SOD1) or to extracellular spaces (EC SOD or SOD3). SOD2 is localized to mitochondria and has Mn as the prosthetic group. SOD1 has a molecular mass of about 32 kDa and has been found in the cytoplasm, nuclear components and lysosomes of mammalian cells (Chang *et al.,* 1988; Keller *et al.,* 1991). SOD1 is a constitutively expressed gene, localized on chromosome 21(21q22) in humans. In addition, over 100 distinct SOD1 inherited mutations have been identified in the familial form of amyotrophic lateral sclerosis (ALS), a progressive degenerative disease of motor neurons. Despite the fact that SOD1 helps to eliminate toxic reactive species, its mutations in ALS have been described as gain-of-function. The mechanism by which mutant SOD1 induces the neurodegeneration observed in ALS is still unclear. Mutant SOD1 proteins become misfolded and consequently oligomerize into high molecular weight species that aggregate and end up in proteinaceous inclusions.

There are several reports which suggest that SOD1 activity is increased in vitiligo patients compared to controls (Hazneci *et al.*, 2005, Yildirim *et al.*, 2003, 2004; Dell'Anna *et al.*, 2001; Chakraborty *et al.*, 1996; Ines *et al.*, 2006). Increased activity of SOD1 results in overproduction of H_2O_2 which is toxic to the cell. Our earlier studies showed impairment in the systemic antioxidant system in Gujarat vitiligo patients (Agrawal *et al.*, 2004, Shajil and Begum, 2006).

The objectives of this study were:

i.) To estimate lipid peroxidation (LPO) levels to assess oxidative stress in vitiligo patients and controls.

ii.) To estimate Superoxide dismutase 1 activity in vitiligo patients and controls.

iii.) To determine *SOD1* mRNA and protein levels in vitiligo patients and controls.

iv.) To investigate exon 2 C/T (Ile40Thr) single nucleotide polymorphism (SNP) of *SOD1* gene in vitiligo patients and controls.

v.) To identify novel mutations/SNPs in *SOD1* gene using High Resolution Melt Curve (HRM) analysis.

2.2 MATERIALS AND METHODS

2.2.1 Study Subjects:

The study group included 950 vitiligo patients comprised of 408 males and 542 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 1650 ethnically, age and sex-matched unaffected individuals comprised of 742 males and 908 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.

2.2.2 Blood collection:

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). The blood was used for Genetic analysis (DNA and RNA preparation) and Biochemical analysis (estimation of LPO, SOD1 and quantification of SOD1 protein by western analysis) (Figure 1).

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

	Vitiligo Patients	Controls
	(n = 950)	(n = 1650)
Average age (mean age ± SD)	31.35 ± 14.28 yrs	29.42 ± 13.12yrs
Sex: Male	408 (42.95%)	742 (44.97%)
Female	542 (57.05%)	908 (55.03%)
Onset age (mean age ± SD)	22.32 ± 13.41 yrs	NA
Duration of disease (mean ± SD)	6.8 ± 5.2 yrs	NA
Type of vitiligo		
Generalized	684 (72.00)	NA
Localized	266 (28.00)	NA
Active vitiligo	705 (74.21)	NA
Stable vitiligo	245 (25.79)	NA
Family history	125 (13.16%)	NA

2.2.3 Estimation of LPO levels and SOD1 activity:

Sample preparation

Whole blood was centrifuged at 3000 rpm for 10 minutes. Plasma was separated and stored in deep freezer (-20°C) until use. Erythrocyte sediment was washed thrice with PBS and hemolysate was prepared by adding distilled water corresponding to the amount of plasma separated.

Hemoglobin estimation: Hemoglobin estimation was done by cyanmethemoglobin method (Dacie and Lewis 1968).

Principle

Drabkin's reagent (ferricyanide) converts the hemoglobin to cyanmethemoglobin (CMG) and the absorbance of CMG is proportional to the hemoglobin concentration. The optical density was measured at 540 nm against distilled water.

Reagents

Drabkin's reagent
Cyanmethemoglobin standard (65mg/dl)

Protocol

Reagent	Blank	Control	Test
CMG standard	-	3 ml	-
Sample	-	-	20 µL
Drabkin's reagent	5 ml	-	5 ml

Mixed well and kept for 5 minutes. Read the absorbance of the test and CMG standard against distilled water at 540 nm separately.

Calculation

$$\frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \frac{251}{1000} \times 65$$

251 is the dilution factor

1000 is to convert mg/dl to gm/dl

Unit: gHb/ dl (gram hemoglobin/100 ml)

2.2.3.1 Lipid Peroxidation (LPO) levels:

Erythrocyte lipid peroxidation was estimated according to the procedure of Beuge and Aust (1978).

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substance (TBARS) which gives a characteristic pink color that can be measured colorimetrically at 532 nm.

Reagents:

PBS (pH 7.4)	0.1 M
TBA Reagent	TBA 100mg, EDTA 46 mg, 20% TCA 5ml, 2.5 N HCl 5ml and total volume was made up to 20 ml with distilled water
Tetra Methoxy Propene (TMP)	10mM, as standard

Sample preparation:

RBC pellet was prepared after centrifugation of the whole blood at 3000 rpm for 10 minutes. RBC pellet was washed thrice with PBS. 40µL of RBC pellet was added to 960µL of distilled water and shaked well.

Protocol

Reagents	Blank	Test
Sample	-	1.0ml
Distilled water	1.0ml	-
TBA reagent	1.0ml	1.0ml

The tubes were kept in boiling water bath for 20min, cooled under running tap water, centrifuged at 3000 rpm for 15 minutes and read the absorbance of the supernatant at 532nm.

Calculation: Calculation was done according to the slope calculated from the standard graph of TMP

$$\frac{\text{OD of the Test}}{\text{Slope}} \times \frac{100}{\text{gHb}}$$

Units – nmoles of MDA formed/ g Hb

2.2.3.2 Superoxide Dismutase 1 (SOD1) estimation:

The estimation of SOD1 activity in erythrocytes was carried out by the method of Marklund and Marklund (1974) with slight modification utilizing the inhibition of auto-oxidation of pyrogallol by SOD1 enzyme. Erythrocyte sediment was washed thrice with PBS. Hemolysate was prepared with hemoglobin concentration of about 1g Hb/dl. 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 2µL of 1:10 diluted erythrocyte lysate as an enzyme source.

Principle:

The autooxidation of pyrogallol, under alkaline condition generates free oxygen radicals which are used by SOD1 present in hemolysate. Decrease in autooxidation shows indirect evidence of SOD1 activity.

Reagents:

1. Tris buffer 100mM (pH 8.2)
2. Pyrogallol (0.2 mM) dissolved in 0.5 N HCl

Protocol:

The 1g Hb/dl. of hemolysate 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
Hemolysate (1g Hb/dl.)	-	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 SOD1 activity was expressed in units/ gHb/min. One unit of SOD1 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

2.2.4 Determination of *SOD1* and *GAPDH* mRNA expression:

2.2.4.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- 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).

2.2.4.2 Real-time PCR:

The expression of *SOD*1 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 melt curve analysis was carried out on the product formed (Figures 3C & D). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

2.2.5 Western blot analysis of SOD1 and GAPDH:

The hemolysate containing 0.5 g% Hb was run on 15% SDS PAGE under reducing conditions (160 mM DTT) along with the low MW protein marker and electro blotted onto PVDF membrane. The membranes were cut into two pieces above cytochrome c (14 kDa) protein band i.e. the upper piece contained GAPDH (37 kDA) and the lower piece contained SOD1 (16 kDa). The membranes were blocked with 1% BSA in PBS for two hours, added to the lower membrane piece anti superoxide dismutase 1 (Cu/Zn) sheep polyclonal Ab (Calbiochem, Germany) diluted to 1: 5000 with PBS containing 0.1% Tween 20; added to the upper membrane piece anti-GAPDH Rabbit polyclonal Ab (Sigma, USA) diluted 1:10000 with PBS containing 0.1% Tween 20; and both the membrane pieces were incubated for two hours at room temperature. Washed three times with PBS containing 0.2% Tween 20; and added to the lower membrane piece 1:5000 diluted (PBS containing 0.1% Tween 20) secondary antibody (Anti-sheep IgG, Sigma, USA); added to the upper membrane piece 1:3000 diluted (PBS containing 0.1% Tween 20) secondary antibody (Anti-Rabbit IgG, Bangalore Genei, India); and incubated for 1 hour at room temperature. Washed both the membrane pieces thrice with PBS containing 0.15% Tween 20, and developed with

DAB (Diaminobenzidine tetrahydrochloride) in 5 ml PBS containing 50 µL of 50mM $NiCl_2$ and 5µL of 30% H_2O_2.

Reagents for SDS PAGE and Immunoblotting:

Acrylamide monomer solution (25 ml)	
Acrylamide	7.3 g
Bis acrylamide	200 mg
Double distilled water	25 ml

Running gel buffer, pH 8.8 (4X, 50 ml)	
Tris Base (1.5 M)	9.08 g

Stacking gel buffer, pH 6.8 (4X, 50ml)	
Tris Base (0.05 M)	3 g

Tank buffer, pH 8.3 (500 ml)	
Tris Base (0.025 M)	1.52 g
Glycine	7.20 g
SDS 0.1%	5 ml from 10% SDS stock solution

Treatment buffer (2 ml)	
Stacking gel buffer	500 µL
SDS 10%	800 µL
Glycerol	400 µL
2 Mercaptoethanol	200 µL
Bromophenol blue	4 mg
Double distilled water	200 µL

Water saturated butanol	
Butanol	50 ml
Double distilled water	5 ml

After shaking well used the top phase

Running gel, 8% (5ml)	
30% acrylamide solution	1.3 ml
Running gel buffer	1.3 ml
10% SDS	50 µL
10% APS	50 µL
TEMED	3 µL
Double distilled water	2.3 ml

Stacking gel, 5% (4ml)	
30% acrylamide solution	670 µL
Double distilled water	2.7 ml
Stacking gel buffer	500 µL

10% SDS	40 µL
10% APS	40 µL
TEMED	4 µL

Transfer buffer (900 ml)	
Tris	2.72 g
Glycine	12.96 g
Double distilled water	720 ml
Methanol	180 ml

2.2.6 Genomic DNA preparation:

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.

2.2.7 Genotyping of *SOD1* C/T (Ile40Thr) polymorphism:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype Ile40Thr (C/T) polymorphism of *SOD1* 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, 57°C 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 enzyme *Acu*I (New England Biolabs, Beverly, MA) was used for digesting amplicons of Ile40Thr (C/T) polymorphism of *SOD1* gene (Table 2). 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 gel 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).

Table 2. Primers and restriction enzymes used for *SOD1* C/T (Ile40Thr) SNP genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs1804450)				*Acu*I
*SOD*1 C/T F	CAGCCTGGGATTTGGACACAGA	57	360	(295 &
*SOD*1 C/T R	GTATGGGTCACCAGCACAGCA			65 bp)
*SOD*1 expression F	CGAGCAGAAGGAAAGTAATGGACCA	65	110	-
*SOD*1 expression R	AGCCTGCTGTATTATCTCCAAACT			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expression R	CAAATGAGCCCCAGCCTTCT			

2.2.8 High Resolution Melting (HRM) Analysis of *SOD1* gene:

Genomic DNA isolated from blood was used to amplify individual exons of *SOD*1 gene using the oligonucleotide primers (Table 3) using High Resolution Melting (HRM) technique on a Real time PCR machine. Real-time PCR was performed in 20 µL volume using LightCycler®480 HRM 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,

primer dependent annealing 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 high resolution melting curve analysis was carried out on the product formed as per the manufacturer's instructions.

Table 3. Primers used for HRM analysis of *SOD1* gene.

*SOD*1 Gene	Forward Primer	Reverse Primer
Exon 1	5'AGTCATTCCCGGCCACT CGCGAC3'	5'CGGCCTCGCAAACAAGCC TCCGTC3'
Exon 2	5'GAGGACACAGGCCTAGA GCAG3'	5'CAGCACAGCACACCC ACCT3'
Exon 3	5'GCTTATCCCAGAAGTCG TGATGC3'	5'GCAAAGGTGGGGGAAACA CGG3'
Exon 4	5'GTGGCATCAGCCCTAAT CCATCTG3'	5'CTGCAAGTACAGTTTATCT GGATC3'
Exon 5 Fragment 1	5'CATCTTTTGGGTATTGTT GGGAGG3'	5'GGATACATTTCTACAGCTA GCAGG3'
Exon 5 Fragment 2	5'CCTGCTAGCTGTAGAAA TGTATCC3'	5'CTAAATCTGTTCCACTGAA GCTG3'

2.2.9 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *SOD1* C/T (Ile40Thr) polymorphism 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 *SOD*1 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 0.05 were considered as statistically significant. LPO levels, SOD1 activity, relative expression of *SOD1* and SOD1 protein levels 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).

2.3 RESULTS

2.3.1 Estimation of lipid peroxidation (LPO) levels in vitiligo patients and controls:

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 (Figure 1A). Moreover, vitiligo subgroups also showed significant difference in LPO levels. Generalized vitiligo patients had higher levels of LPO as compared to localized vitiligo (p=0.001) (Figure 1A). Interestingly, when LPO levels were analyzed based on progression of the disease, patients with active vitiligo showed significantly higher LPO levels as compared to stable vitiligo (p<0.0001) (Figure 1B).

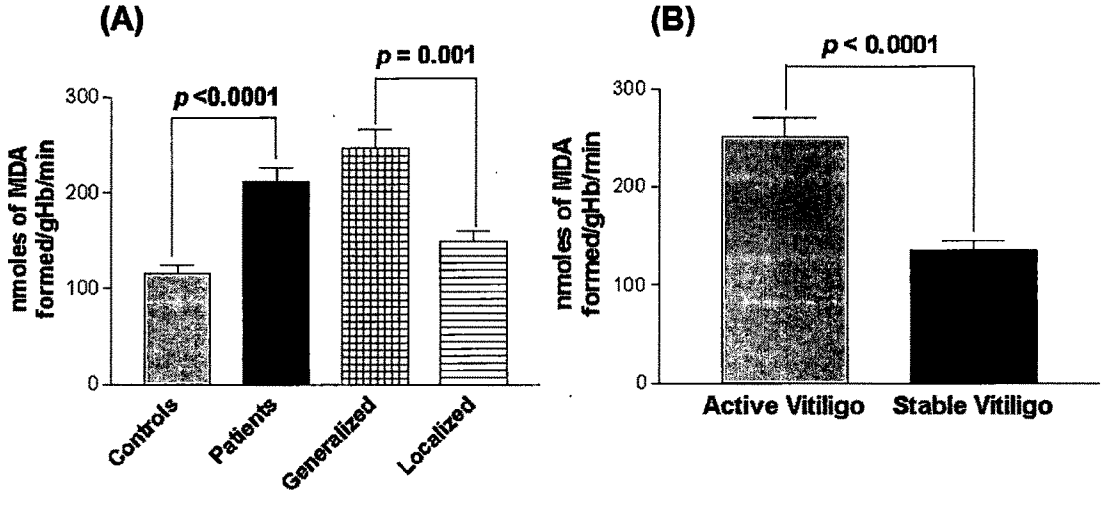


Figure 1. Lipid peroxidation (LPO) levels in controls and vitiligo patients:

(A) LPO levels in 950 vitiligo patients and 1650 controls. Vitiligo patients showed significantly increased LPO levels as compared to controls (Mean ± SEM: 211.6 ± 14.90 vs 115.6 ± 8.949; p<0.0001). Generalized vitiligo patients (n=684) showed

significantly higher LPO levels as compared to localized patients (n=266) (Mean ± SEM: 246.3 ± 20.58 vs 149.5 ± 11.16; p=0.001).

(B) LPO levels with respect to progression of the disease in 705 patients with active and 245 patients with stable vitiligo. Active vitiligo patients showed significantly higher LPO levels as compared to stable vitiligo patients (Mean ± SEM: 251.2 ± 19.55 vs 135.8 ± 9.874; p=0.026).

2.3.2 Estimation of Superoxide Dismutase 1 activity in vitiligo patients and controls:

The estimation of SOD1 activity in erythrocytes was carried out in 950 vitiligo patients and 1650 controls. The SOD1 activity was significantly (p<0.0001) higher in vitiligo patients as compared to controls (Figure 2A). Moreover, vitiligo subgroups also showed significant difference in SOD1 activity. Generalized vitiligo patients had significantly increased SOD1 activity as compared to localized vitiligo (p=0.0003) (Figure 2A). Interestingly, when SOD1 activity was analyzed based on progression of the disease, patients with active vitiligo showed significantly higher SOD1 activity as compared to stable vitiligo (p=0.0006) (Figure 2B).

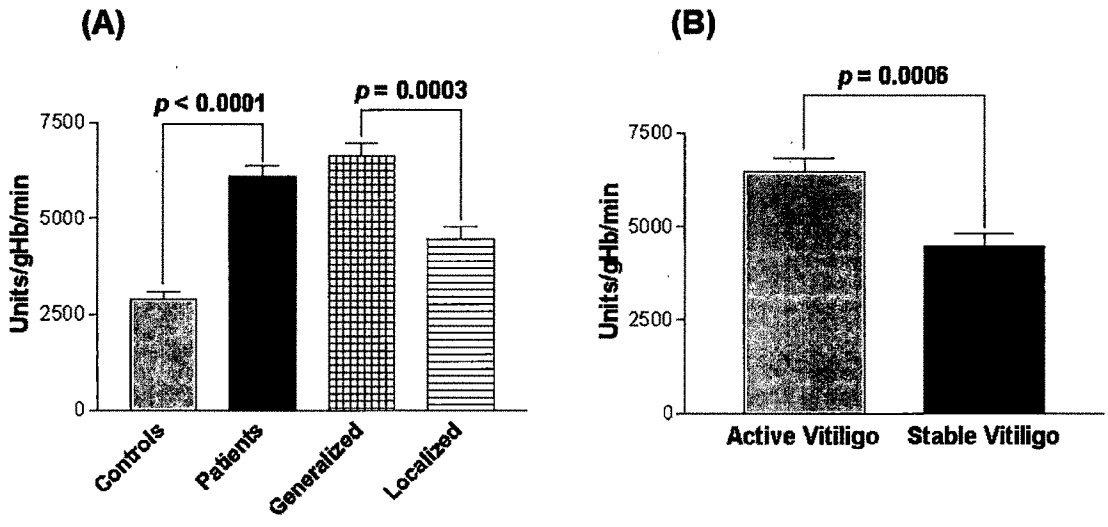


Figure 2. SOD1 activity in controls and vitiligo patients:

(A) SOD1 activity in 950 vitiligo patients and 1650 controls. Vitiligo patients showed significantly increased SOD1 activity as compared to controls (Mean ± SEM: 6088 ± 283.7 vs 2870 ± 193.5; p<0.0001). Generalized vitiligo patients (n=684) showed

significantly increased SOD1 activity as compared to localized patients (n=266) (Mean ± SEM: 6607 ± 365.2 vs 4452 ± 320.1; p=0.0003).

(B) SOD1 activity with respect to progression of the disease in 705 patients with active and 245 patients with stable vitiligo. Active vitiligo patients showed significantly increased SOD1 activity as compared to stable vitiligo patients (Mean ± SEM: 6471 ± 356.4 vs 4440 ± 378.2; p=0.0006).

2.3.3 Determination of SOD1 mRNA and protein levels in vitiligo patients and controls:

2.3.3.1 Relative gene expression of SOD1 in patients with vitiligo and controls:

*SOD1*gene expression studies showed no difference in expression of *SOD1* transcripts in 166 vitiligo patients compared to 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p=0.820) (Figure 3A). Moreover, there was no significant difference in expression of *SOD1* between generalized and localized vitiligo patients (p=0.250) (Figure 3A). In addition, we also checked the effect of *SOD1* expression on progression of the disease i.e. active and stable cases of vitiligo (Figure 3B); however, no significant difference was observed between active and stable vitiligo patients (p=0.970). The specificity of the product formed for SOD1 and GAPDH was checked by melt curve analysis (Figure 3C & D).

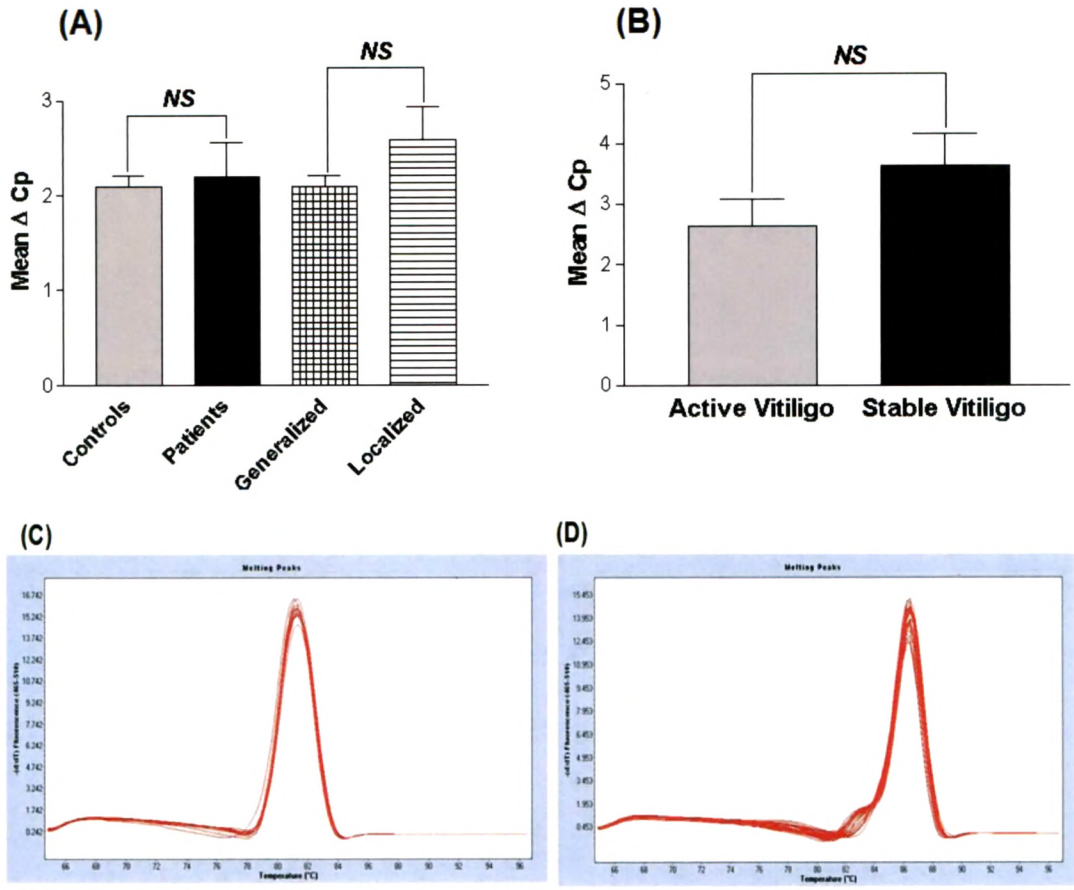


Figure 3. Relative gene expression of *SOD1* in controls and vitiligo patients:

(A) Expression of *SOD1* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients did not show significant difference in mRNA levels of *SOD1* as compared to controls (Mean ΔCp ± SEM: 2.193 ± 0.3586 vs 2.090 ± 0.1173; p=0.820). No significant difference was observed for *SOD1* mRNA levels between generalized and localized vitiligo patients (Mean ΔCp ± SEM: 2.032 ± 0.1164 vs 2.581 ± 0.3518; p=0.250).

(B) Expression of *SOD1* 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. No significant difference was observed for *SOD1* mRNA levels between active and stable vitiligo patients (Mean ΔCp ± SEM: 2.457 ± 0.4170 vs 2.479 ± 0.3443; p=0.970).

(C) & **(D)** Melt curve analysis of *SOD1* and *GAPDH* showing specific amplification.

2.3.3.2 SOD1 protein levels in patients with vitiligo and controls by western blot analysis:

SOD1 protein levels were monitored in human erythrocytes of 50 control subjects and 50 Gujarat vitiligo patients by western blot using GAPDH as an internal control (Figure 4 & 5). However, no significant difference was observed in SOD1 protein levels using densitometric analysis between patients and controls as suggested by mean IDV (integrated density value) (p=0.659; Figure 5A). Moreover, generalized and localized vitiligo groups did not show significant difference in SOD1 protein levels (p=0.214; Figure 5A). Also, no significant difference in SOD1 protein levels was observed between patients with active and stable vitiligo (p=0.256; Figure 5A).

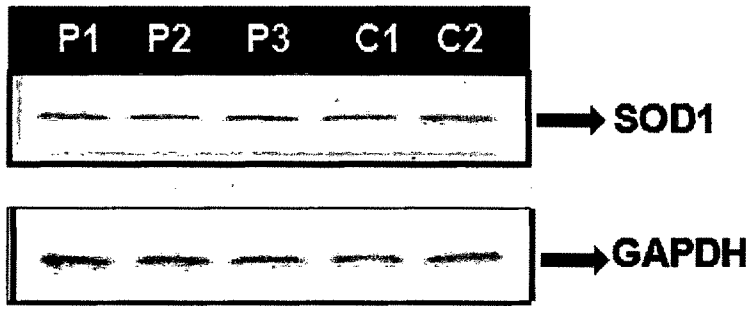


Figure 4. Western blot analysis of erythrocyte SOD1 and GAPDH protein levels in vitiligo patients and controls: Representative western blot for SOD1 and GAPDH protein levels showing vitiligo patients as P1, P2, P3 and C1, C2 as healthy controls.

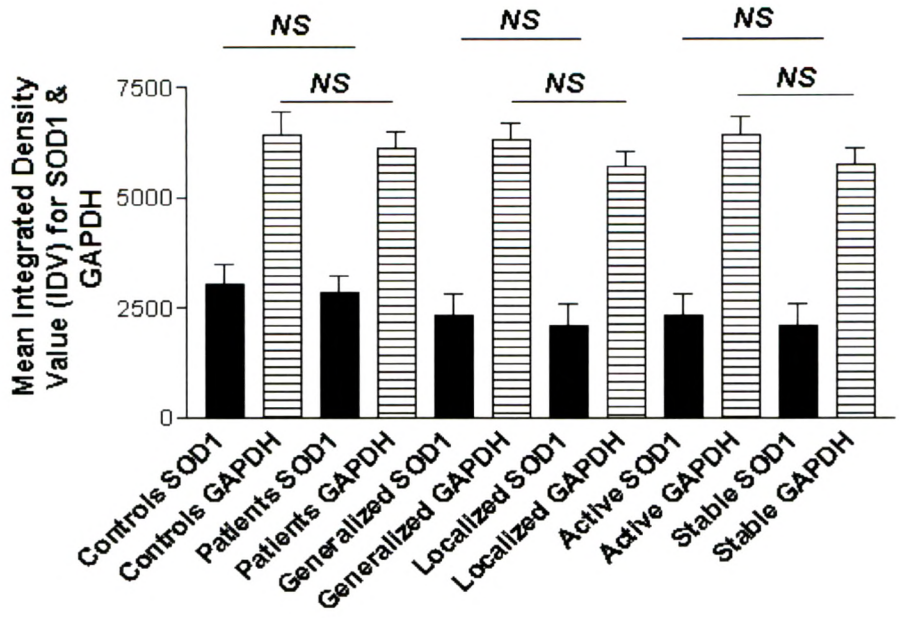


Figure 5. Densitometric analysis of SOD1 and GAPDH protein levels in controls and vitiligo patients:

GAPDH protein levels (as IDV) in 50 vitiligo patients and 50 controls. No significant difference was observed in SOD1 protein levels between vitiligo patients and controls (Mean ± SEM: 6130 ± 374.9 vs 6425 ± 530.0; p=0.659). No significant difference was observed in SOD1 protein levels between generalized (n=32) and localized (n=18) vitiligo patients (Mean ± SEM: 6307 ± 361.7 vs 5701 ± 322.1; p=0.214). No significant difference was observed in SOD1 protein levels between active (n=35) and stable (n=15) vitiligo patients (Mean ± SEM: 6404 ± 439.1 vs 5753 ± 364.5; p=0.256).

SOD1 protein levels (as IDV) in 50 vitiligo patients and 50 controls. No significant difference was observed in GAPDH protein levels between vitiligo patients and controls (Mean ± SEM: 2854 ± 382.7 vs 3026 ± 455.4; p=0.776). No significant difference was observed in GAPDH protein levels between generalized (n=32) and localized (n=18) vitiligo patients (Mean ± SEM: 2333 ± 475.9 vs 2110 ± 495.4; p=0.749). No significant difference was observed in GAPDH protein levels between active (n=35) and stable (n=15) vitiligo patients (Mean ± SEM: 2339 ± 459.1 vs 2082 ± 515.8; p=0.713) (NS=Non-significant).

2.3.4 Analysis of association between *SOD1* exon 2 C/T (Ile40Thr) polymorphism and susceptibility to vitiligo:

PCR-RFLP for *SOD1* C/T (Ile40Thr) polymorphism yielded a 360 bp undigested product corresponding to C allele, 295 bp and 65 bp digested products corresponding to T allele. However, only one genotype 'CC' was identified by 2.5 % agarose gel electrophoresis (Figure 6).

The *SOD1* C/T (Ile40Thr) polymorphism was not found to be associated with vitiligo susceptibility (p=1.00) (Table 4). Interestingly, the polymorphism was found to be monogenic; being only CC alleles presented in control and both the patient groups (Table 4). Also, the distribution of genotype frequencies for *SOD1* C/T (Ile40Thr) polymorphism was not consistent with Hardy-Weinberg expectations in both patient and control groups (p<0.0001) (Table 4).

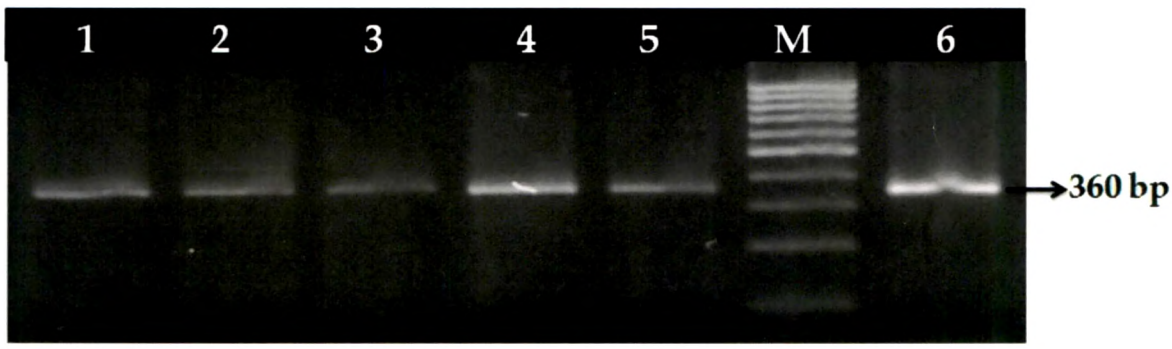


Figure 6. PCR-RFLP analysis of *SOD1* C/T (Ile40Thr) polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 2, 3, 4, 5 & 6 show individuals with homozygous CC genotypes; lane M shows 100 bp DNA ladder. No individual with homozygous TT and heterozygous CT was found.

Table 4. Association study for *SOD1* C/T (Ile40Thr) polymorphism in vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE
	Genotype	(n = 285)	(n = 300)		
	CC	285 (1.00)	300 (1.00)		<0.0001[a]
SOD1	CT	00 (0.00)	00 (0.00)	1.000	(P)
C/T	TT	00 (0.00)	00 (0.00)		
(rs1804450)	Allele				<0.0001[b]
	C	570 (1.00)	600 (1.00)	1.000	(C)
	T	00 (0.00)	00 (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,

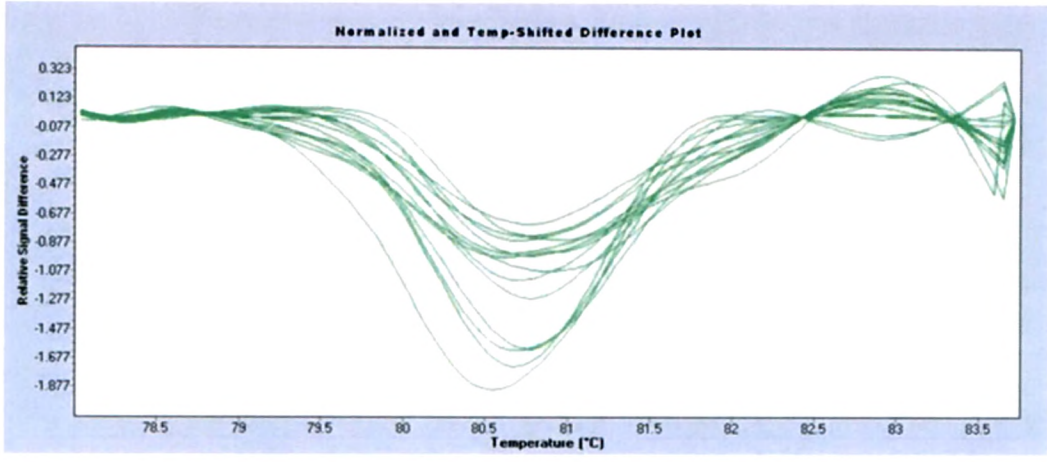
[b] Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table,

Values are significant at $p \leq 0.05$.

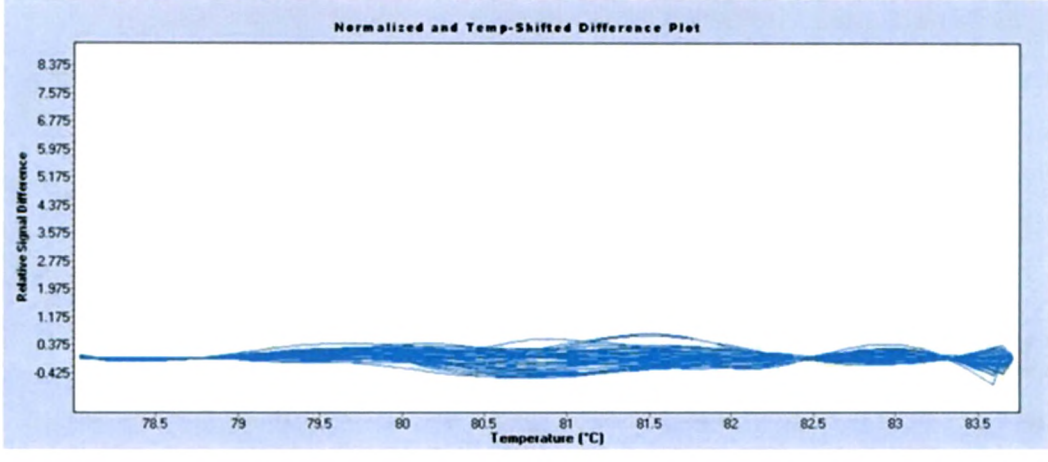
2.3.5 *SOD1* gene scanning for novel genetic variations using High Resolution Melt Curve (HRM) analysis:

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 (Figure 7). 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 (Figure 8).

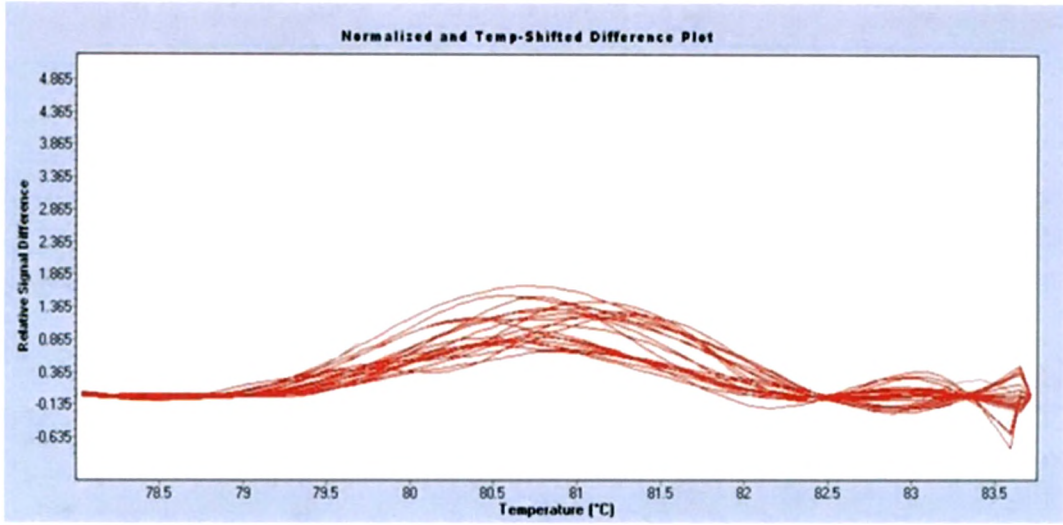
(A) Exon 1



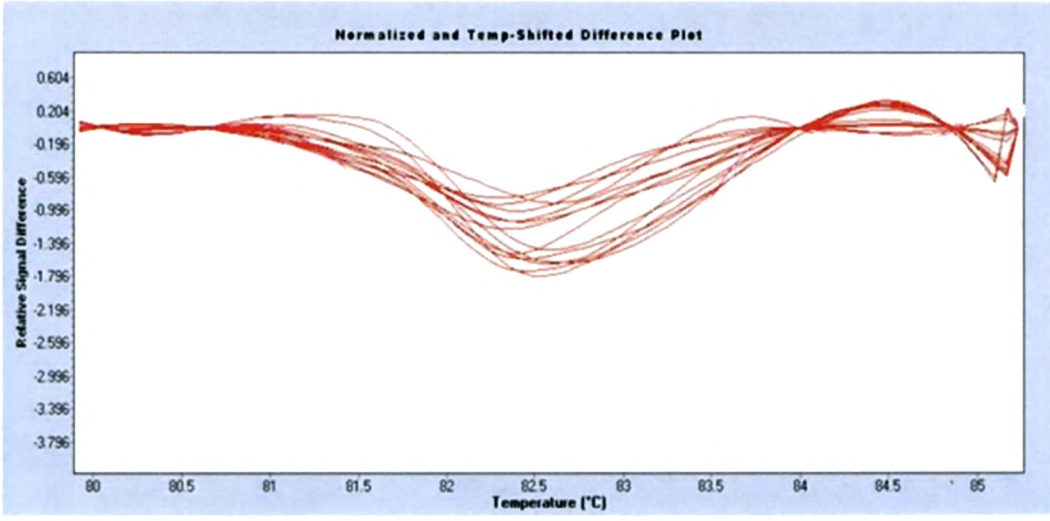
(B) Exon 2



(C) Exon 3



(D) Exon 4



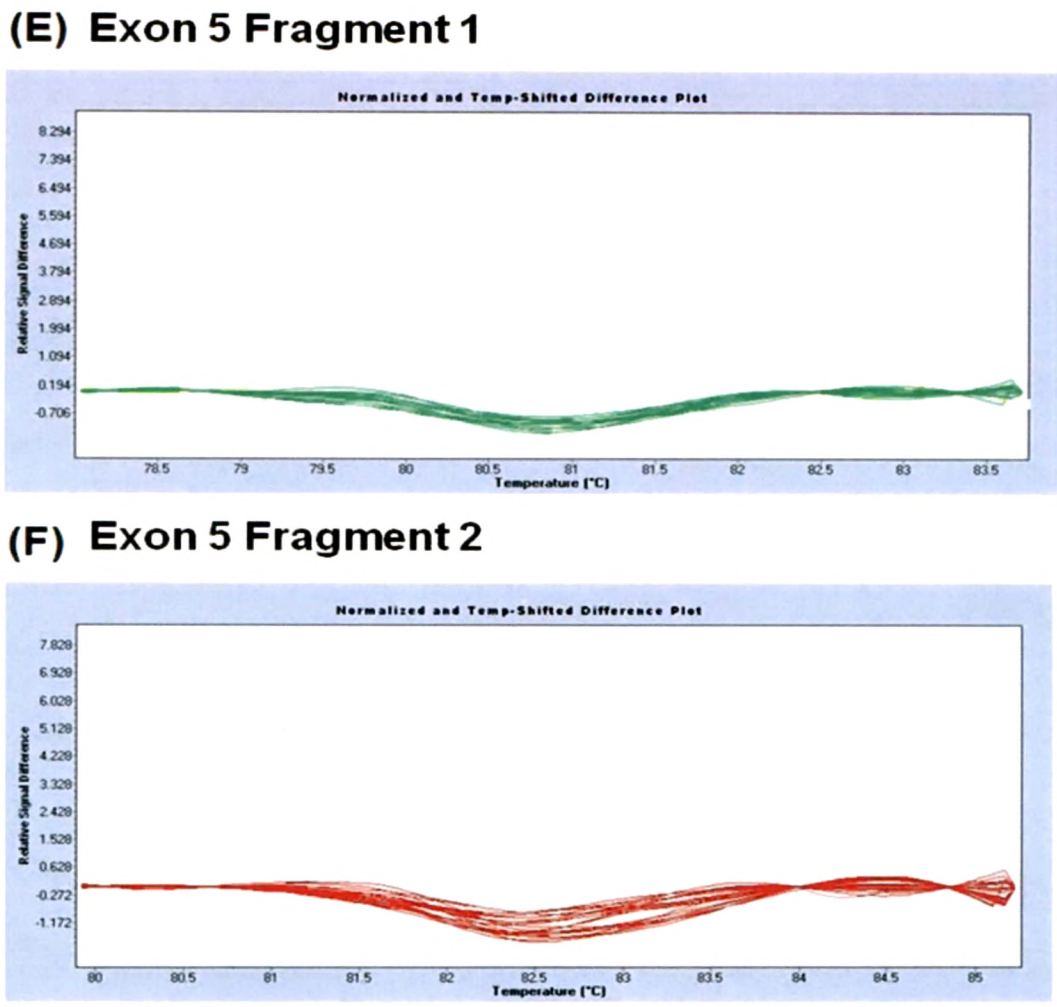


Figure 7. HRM analysis of *SOD1* gene in vitiligo patients and controls.

Figure 8. Representative BLAST analysis for *SOD1 sequence from* vitiligo patient showing 99% homology with the wild type sequence.

2.4 DISCUSSION

Oxidative stress acting as the triggering event in melanocyte degeneration is well established (Picardo *et al.,* 1994; Passi *et al.,* 1998). Different molecular events which lead to the accumulation of hydrogen peroxide are well documented (Schallreuter *et al.,* 1994; 1996; Rokos *et al.,* 2002; Gibson and Lilley, 1997; Kaufman, 1997; Dell'Anna *et al.,* 2001; Maresca *et al.,* 1997; Beazley *et al.,* 1999; Jimbow *et al.,*

2001; Schallreuter *et al.*, 1991). Hydrogen peroxide thus formed further inhibits epidermal catalase resulting in oxidative stress, which leads to the destruction of melanocytes (Schallreuter *et al.*, 1999). Low epidermal catalase levels in both lesional and non-lesional epidermis of vitiligo patients suggests that the entire epidermis may be involved in this disorder (Schallreuter *et al.*, 1999). Oxidative stress hypothesis also considers a systemic involvement during the course of the disease (Boisseu-Garsuad *et al.*, 2002; Picardo *et al.*, 1994; Yildirim *et al.*, 2003; Agrawal *et al.*, 2004). Thus oxidative stress could act as the initial triggering event in melanocyte degeneration. Local/systemic factors affect the homeostasis of the epidermal melanin unit in segmental vitiligo whereas an impaired redox status of the epidermal melanin unit acts as the primary defect further leading to inappropriate immune response in non-segmental vitiligo (Taieb, 2000).

The present study showed significant increase in LPO levels in patients compared to controls suggesting high oxidative stress in vitiligo patients (Figure 1A). Our results are in line with earlier studies (Agrawal *et al.*, 2004; Shajil and Begum, 2006, Shajil *et al.*, 2007; Khan *et al.*, 2009). Moreover, LPO levels were high in patients with generalized vitiligo as compared to localized, suggesting higher level of oxidative stress in generalized cases (Figure 1A). Analysis based on the progression of disease also showed increased oxidative stress in active cases compared to stable cases of vitiligo as LPO levels were significantly increased in active cases implicating the role of oxidative stress in progression of disease (Figure 1B).

Superoxide dismutase scavenges the superoxide radicals and reduces its toxicity (McCord and Fridovich, 1969). In the present study significant increase in erythrocyte SOD1 activity was observed in vitiligo patients (Figure 2A). Interestingly, generalized vitiligo patients in the present study exhibited increased SOD1 activity compared to localized group of patients suggesting the differential role of SOD1 activity in manifestation of vitiligo in different types (Figure 2A). Also an increase in SOD activity was reported by Chakraborty *et al.* (1996), Yildirim *et al.* (2003) and Agrawal *et al.* (2004). However, Picardo *et al.* (1994) reported that SOD activity in erythrocytes of vitiligo patients was not significantly different from the healthy age matched controls (Picardo *et al.*, 1994). Passi *et al.* (1998) also showed that there is no significant change in the epidermal SOD levels in vitiligo patients compared to

controls (Passi *et al.*, 1998). Nevertheless, significant decrease in serum SOD levels was reported by Koca *et al.* (2004) in generalized vitiligo. Maresca *et al.* (1997) have observed no difference in the SOD activity in cultured vitiliginous melanocytes compared to cultured melanocytes of normal subjects. The increased activity of SOD1 was consistently high in active cases of vitiligo as compared to stable cases suggesting that SOD1 has an important role in progression of disease (Figure 2B). Our results are in line with those of Jain *et al.* (2011) suggesting increased SOD1 activity in active cases of vitiligo in Indian population. Previously, Sravani *et al.* (2009) reported a significant increase in the levels of SOD in vitiliginous and non vitiliginous skin of patient group compared to the control group.

Further, the study was aimed to verify whether the increase in SOD1 activity in vitiligo patients is due to increased mRNA and/or protein levels, relative gene expression and western blot analysis of SOD1 was carried out. The relative gene expression of *SOD1* suggested no significant difference in *SOD1* mRNA levels in patients and controls (Figure 4A). This study was further extended for generalized, localized, active and stable cases of vitiligo which also could not find significant difference in *SOD1* mRNA levels between these groups (Figure 4A & B). Moreover, to confirm the *SOD1* expression results western blot analysis was performed and the results suggested no significant difference in SOD1 protein levels in patients as compared to controls (Figure 5 & 6A). Also the SOD1 protein levels did not differ in generalized, localized, active and stable groups of vitiligo patients (Figure 6 A). These two expression studies of *SOD1* confirm that the increased activity of SOD1 in patients as well as different types of vitiligo was not due to increased mRNA and/or protein levels. There are no reports available which showed increased activity of SOD1 without a change in its protein content.

We speculated that the increased activity of SOD1 observed in patients might be due to genetic varaints present in *SOD1* gene exonic regions, as possibility of promoter variants was nullified due to non-significant difference in mRNA and protein levels of SOD1. Thus our results suggested that mutation/s in the exonic regions of *SOD1* gene may enhance the activity of this enzyme. One of the reported SNP in *SOD1* gene is exon 2 C/T (Ile40Thr) which substitutes Ile to Thr. We investigated this polymorphism to find its association with vitiligo; however, the SNP remained

uninformative as only single 'CC' wild type genotype was observed in both control and patient groups (Table 4). This result led us to screen the exonic regions of *SOD1* gene which may harbor novel mutations responsible for increased SOD1 activity. However, *SOD1* gene scanning through high resolution melting (HRM) curve analysis could not find any mutation in the five exonic regions of *SOD1* gene in patients. These results further suggest that increased SOD1 activity may be due to post translational modifications of SOD1.

From the current study, it is clear that increased activity of erythrocyte SOD could enhance the systemic production of H_2O_2 which affects the downstream antioxidant enzymes that neutralize H_2O_2 i.e. catalase and glutathione peroxidase as observed in vitiligo patients (Agrawal *et al.*, 2004). Low levels of catalase or/and GPX in patients could result in excessive production of H_2O_2, which in turn leads to oxidative stress as evident by high LPO levels in vitiligo patients.

In conclusion, impairment of the systemic antioxidant system due to increased SOD1 activity results in oxidative stress in vitiligo patients indicating that melanocyte damage in vitiligo may be linked to generalized oxidative stress. Further, the study proposes that post translational modifications may play an important role in increasing the efficiency of SOD1.

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