CHAPTER IV

ROLE OF SUPEROXIDE DISMUTASE 3 (SOD3) IN VITILIGO SUSCEPTIBILITY

4.1 INTRODUCTION:

Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction (Schallreuter *et al.,* 1999; Schallreuter *et al.,* 1994a) as H_2O_2 accumulation is observed in the epidermis of active vitiligo patients (Hasse *et al.,* 2004). 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 reactive oxygen species 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. Previously, systemic oxidative stress in vitiligo patients of Gujarat due to an imbalance in enzymatic and non-enzymatic antioxidant systems has been reported (Agrawal *et al.,* 2004; Shajil and Begum, 2006).

Reactive oxygen species are produced in the body by several different mechanisms, including cellular respiration, interactions between ionizing radiation and biological molecules, and phagocytosis (McCord, 1993). ROS produced at lower levels under normal conditions are important mediators of cell signaling events, including differentiation, cell cycle progression, growth arrest, apoptosis, and immunity (Oury *et al.,* 1991). Under physiological conditions, a balance exists between the level of ROS produced during normal cellular metabolism and the level of endogenous antioxidants, which serve to protect tissues from oxidative damage. Disruption of this balance, either through increased production of ROS or decreased levels of antioxidants, produces a condition referred to as oxidative stress and leads to variety of pathological conditions including cardiovascular diseases, neurological disorders, lung pathologies, accelerated aging and UV-induced skin inflammation (Bowler and Crapo 2002; Delanty and Dichter, 1998; Fukai *et al.,* 2002). Superoxide radicals produced from a one-electron reduction of oxygen can undergo either spontaneous or enzyme-catalyzed dismutation to hydrogen peroxide (H_2O_2) or can react with nitric oxide (NO) to form the toxic product peroxynitrite ($ONOO^-$). Either the combination of H_2O_2 with metal ions (iron) or the breakdown of $ONOO^-$ can produce the highly

toxic hydroxyl radical (•OH). These ROS can react with a variety of cellular macromolecules such as lipids, proteins and DNA leading to the disruption of cell membranes, inappropriate activation or inactivation of enzymes, and genetic mutations. Therefore, persistence of these diverse ROS in and around cells and tissues can have severe pathophysiological consequences including vitiligo.

The antioxidant enzyme superoxide dismutase (SOD) family comprises three distinct isoforms i.e. SOD1, SOD2 and SOD3 that are responsible for maintaining low levels of ROS by metabolizing superoxide anion into oxygen and hydrogen peroxide. The SOD3 is an extracellular (EC SOD) (Marklund *et al.*, 1982) enzyme encoded on chromosome 4p16.3-q21. Human EC-SOD is a metalloprotein containing copper and zinc ions in its active site, which support enzymatic activities. EC-SOD contains four functional domains that include a signal peptide (amino acid residues 1-18), an amino-terminal domain (residues 19–95) with a glycosylation site, a central domain with active site that shows 50% homology to Cu/Zn SOD (residues 96–193) with binding sites for copper and zinc ions, and a heparin- binding domain at its carboxyl terminus (residues 194–222). EC-SOD exists as a tetramer composed of two interacting dimers, and the tetramer is held together by van der Waals and hydrogen bonding/salt bridge interactions. SOD3 is a secretory protein (Beckman *et al.*, 1994). In mammals, 90-99% of the SOD3 is found in the interstitial spaces of tissues (Kroncke *et al.*, 1994; Squardrito *et al.*, 1995). However, the enzyme is also found in extracellular fluids and accounts for the majority of the SOD activity of plasma, lymph, and synovial fluid (Foalz *et al.*, 1994; Sandsrom *et al.*, 1994). As a copper-containing enzyme, the activity of SOD3 is regulated by copper availability. Free intracellular copper is extremely limited (Rae *et al.*, 1999) and soluble carrier proteins or "copper chaperones" are used for copper trafficking to specific coper containing proteins.

The mature human SOD3 contains six cysteine residues (Hjalmarsson *et al.*, 1987) and occurs naturally in two different forms with evident disulfide bridges (Petersen *et al.*, 2003). One form is active (aSOD3) and has a free cysteine residue at position 195. The intrasubunit disulfide bridge formed by Cys107 and Cys189 is necessary for the SOD3 activity (Petersen *et al.*, 2003). The other form has a free cysteine residue at position 45 and is inactive (iSOD3). SOD3 is unusually resistant to high temperatures,

extreme pH, and high urea concentrations, although it can be inhibited by a variety of agents including azide and cyanide and inactivated by diethyldithiocarbamate and hydrogen peroxide.

SOD3 is also found in the extracellular matrix of mammalian tissues (Marklund, 1984; Oury *et al.*, 1994). The C-terminal region is highly rich in basic amino acid residues (Hjalmarsson *et al.*, 1987). This polybasic region is involved in the binding to heparin/heparan sulfate (Adachi *et al.*, 1992) and type I collagen (Petersen *et al.*, 2004) and is referred to as the extracellular matrix (ECM)-binding region (Petersen *et al.*, 2004). The ECM region is removed proteolytically just before secretion. This process is a two step event involving an initial cleavage of the protein by furin or another member of the proprotein convertase family of procesing proteinases followed by the action of an unknown Arg/Lys-specific carboxypeptidase (Bowler *et al.*, 2002; Enghild *et al.*, 1999; Olsen *et al.*, 2004). A fundamental and distinguishing property of SOD3 is its affinity for certain glycosaminoglycans such as heparin and heparan sulfate. The last 20 amino acids of the 222-amino acids long SOD3 C-type subunit contains 6 arginines, 3 lysines and 1 histidine. The cluster of 6 positively charged amino acids at positions 210-215 forms the essential part of the heparin binding domain (Sandstrom *et al.*, 1992). It binds to the negatively charged molecules: heparin and heparan sulphate (which is of electrostatic nature) (Karlsson *et al.*, 1988) and coverts superoxide anion to H_2O_2. Superoxide anion can easily react with nitric oxide (NO) to form the toxic product peroxynitrite ($ONOO^-$) and SOD3 is important in preserving NO function by maintaining low superoxide concentrations in blood vessels (Fattman *et al.*, 2003). The heparin binding domain has been proposed to act as a nuclear localization signal in certain cell types (Ookawara *et al.*, 2003), suggesting that SOD3 may also provide antioxidant protection to DNA and nuclear proteins.

In *SOD3* gene two missense mutations i.e. Arg213Gly (C/G; rs8192291) and Ala40Thr (G/A; rs2536512), have been reported (Yamada *et al.*, 1997; Yamada *et al.*, 1995). Additional variants of the human *SOD3* gene include three missense mutations: Phe131Cys, Val160Leu, and Arg202Leu. A silent mutation, Leu53Leu (CTG to TTG), has also been found in samples of Japanese and Mediterranean populations (Qin *et al.*, 2008; Campo *et al.*, 2005; Tamai *et al.*, 2006).

The Arg213Gly (C/G) SNP present in exon3 of *SOD3* gene at codon 213 inhibits ionic interactions between heparin and SOD3 which in turn results in 10 fold increase in SOD3 concentration in plasma (Sandstrom *et al.*, 1994). The Arg213Gly SNP located at the heparin-binding domain has been identified in a small proportion of the healthy population (Folz *et al.*, 1994) and has been associated with very high plasma SOD3 levels due to altered affinity of SOD3 for heparin at the endothelial cell surface (Sandstrom *et al.*, 1994; Adachi *et al.*, 1996; Karlsson *et al.*, 1994).
The Ala40Thr polymorphism is located in the amino terminal domain, where it is thought to work for the tetramerization of the enzyme. The findings of Tamai *et al.*, (2006) suggested the possibility that the Thr allele of the Thr40Ala polymorphism is associated with impaired function of SOD3.

However, 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. In the present study, we have made an attempt to understand the role of SOD3 in vitiligo pathogenesis. Hence, the objectives of this study were:

i.) To determine whether the Arg213Gly (C/G; rs8192291) and Ala40Thr (G/A; rs2536512) polymorphisms of *SOD3* are associated with vitiligo susceptibility and modulate SOD3 activity.

ii.) To measure and compare *SOD3* transcript levels and activity in patients with vitiligo and in unaffected controls.

iii.) To correlate *SOD3* polymorphisms, expression levels and its activity with progression of the disease.

4.2 MATERIALS AND METHODS

4.2.1 Study Subjects:

The study group included 482 vitiligo patients comprised of 208 males and 274 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 five hundred and sixty four ethnically, and sex-matched unaffected individuals comprised of 249 males and 315 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.

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

	Vitiligo Patients	Controls
	(n =482)	(n =564)
Average age (mean age ± SD)	32.45 ± 13.48 yrs	28.23 ± 14.42yrs
Sex: Male	208 (43.15%)	249 (44.15%)
Female	274 (56.85%)	315 (55.85%)
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	328 (68.05)	NA
Localized	154 (31.95)	NA
Active vitiligo	352 (73.03)	NA
Stable vitiligo	130 (26.97)	NA
Family history	63 (13.07%)	NA

4.2.2 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.

4.2.3 Genotyping of *SOD3* Arg213Gly (C/G) and Ala40Thr (G/A) polymorphisms:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype Arg213Gly (C/G) and Ala40Thr (G/A) polymorphisms of *SOD3* 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 *Mwo*I and *Pau*I (New England Biolabs, Beverly, MA) were used for digesting amplicons of Arg213Gly (C/G) and Ala40Thr (G/A) polymorphisms of *SOD3* 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 50 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 20% polyacrylamide or 2.5% agarose 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).

Table 2. Primers and restriction enzymes used for *SOD3* Arg213Gly (C/G; rs1799895) and Ala40Thr (G/A; rs2536512) SNPs genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs1799895)				*Mwo*I
SOD3 C/G F	CGCCAGGCGCGGGAACACTCAG	64	63	(35 & 28
SOD3 C/G R	GGCGGACTTGCACTCGCTCTCG			bp)
(rs2536512)				*Pau*I
SOD3 G/A F	GGTTCTGCGATAATGGGGTCCCT	58	396	(274 & 121
SOD3 G/A R	GGCGAAGAAGGCGTCGAGCTT			bp)
SOD3 expression F	GCTGGAAAGGTGCCCGACTC	65	162	-
SOD3 expression R	CAGATCTCCGTGACCTTGGCG			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expression R	CAAATGAGCCCCAGCCTTCT			

4.2.4 Estimation of Superoxide dismutase 3 activity:

The estimation of SOD3 activity in plasma was carried out by the method of Marklund and Marklund (1974) with slight modifications utilizing the inhibition of auto-oxidation of pyrogallol by SOD3 enzyme. The final assay mixture contained 3 ml of tris buffer (200 mM, pH 8.2) containing air equilibrated 0.2 mM pyrogallol [E merck, India], 1 mM EDTA and 2µL of 1:10 diluted plasma as an enzyme source. The concentration of protein in the plasma was estimated by Lowry's method (Lowry *et al.*, 1954) (as described in Chapter III).

Principle:

The autooxidation of pyrogallol, under alkaline condition generates free oxygen radicals which are used by SOD3 present in plasma sample. Decrease in autooxidation shows indirect evidence of SOD3 activity.

Reagents:

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

Protocol:

The 1:10 dilution of plasma 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
Plasma (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 min at 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. The SOD3 activity was expressed in units/mg of plasma protein. One unit of SOD3 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

Unit: One unit of SOD3 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation.

Specific activity: Units of enzyme/ mg of protein

4.2.5 Determination of *SOD3* and *GAPDH* mRNA expression:

4.2.5.1.1 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/ ethidium bromide staining and purity by monitoring 260/280 absorbance ratio of >1.95. 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).

4.2.5.1.2 Real-time PCR:

The expression of *SOD3* 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 2D). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

4.2.6 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for both the 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 *SOD3* 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 0.025 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.

SOD3 specific activity and relative expression of *SOD*3 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).

4.3 RESULTS:

4.3.1 Analysis of association between *SOD*3 C/G; Arg213Gly polymorphism and susceptibility to vitiligo

PCR-RFLP for *SOD*3 C/G Arg213Gly polymorphism yielded a 63 bp undigested product corresponding to G allele and 35 bp and 28 bp digested products corresponding to C allele. The three genotypes identified by 20% polyacrylamide gel electrophoresis were: CC homozygous, CG heterozygous and GG homozygous for Arg213Gly polymorphism of *SOD*3 gene (Figure 1A).

The genotype and allele frequencies of the C/G Arg213Gly polymorphism in 455 vitiligo patients and 548 controls are summarized in Table 3. The Arg213Gly polymorphism of *SOD*3 gene was found to be in significant association with vitiligo patients (p=0.001) when genotypes were compared with chi-squared test-3x2 contingency table (Table 3). The minor allele (G) of Arg213Gly polymorphism was more frequent in the vitiligo group compared to the control group (16.0% versus 8.0%, p=0.001; OR 1.363, 95% CI 1.134- 1.639) consistent with a susceptibility effect (Table 3). Both patient and control populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.881 and p=0.081 respectively) (Table 3).

Moreover, generalized vitiligo group showed significant association of Arg213Gly polymorphism when the genotypes were compared with those of control group

(p=0.003); however localized vitiligo group did not show significant association of this polymorphism (p=0.047) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized vitiligo group when the alleles were compared with those of control group (p=0.004); however, localized vitiligo group showed marginal significance difference in allele frequencies (p=0.021) (Table 4). The distribution of *SOD3* Arg213Gly 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 Arg213Gly 'G' allele occurred prevalently in the group of patients with active vitiligo compared to the control group (41.00% versus 32.00%, p=0.0002) (Table 5). However, there was no statistically significant difference in the distribution of the Arg213Gly 'G' allele between patients with stable vitiligo and control group (34.00% versus 32.00%, p=0.487) (Table 5). Also, the Arg213Gly 'G' allele did not show significant difference between patients with active vitiligo as compared to stable vitiligo (41.00% versus 34.00%, p=0.119) (Table 5). This study has 81.46% statistical power for the effect size 0.8 to detect association of Arg213Gly polymorphism of *SOD3* at p<0.05 in patients and control population.

4.3.2 Analysis of association between *SOD3* G/A; Ala40Thr polymorphism and susceptibility to vitiligo

PCR-RFLP for *SOD3* G/A (Ala40Thr) polymorphism yielded a 396 bp undigested product corresponding to G allele, 274 bp and 121 bp digested products corresponding to A allele. The three genotypes identified by 2.5% agarose gel electrophoresis were: GG homozygous, GA heterozygous and AA homozygous for G/A (Ala40Thr) polymorphism of *SOD3* gene (Figure 1B).

The genotype and allele frequencies of the G/A (Ala40Thr) polymorphism in 448 vitiligo patients and 550 controls are summarized in Table 3. The G/A (Ala40Thr) polymorphism was not found to be in significant association with vitiligo patients as suggested by the genotype and allele frequencies for the polymorphism (p=0.802; p=0.873) (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.070 and p=0.315 respectively)

(Table 3). Also, both generalized and localized vitiligo groups did not show significant association of G/A (Ala40Thr) polymorphism when the genotypes were compared with those of control group (p=0.615; p=0.746 respectively) (Table 4). The distribution of *SOD*3 G/A (Ala40Thr) genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups (p>0.05).

There was no statistically significant difference in the distribution of the Ala40Thr 'A' allele between both the groups of patients with active and stable vitiligo and control group (p=0.816; p=0.304) (Table 5). Also, the Ala40Thr 'A' allele did not show significant difference between patients with active vitiligo as compared to stable vitiligo (p=0.241) (Table 5).

This study has 81.30% statistical power for the effect size 0.08 to detect the association of *SOD*3 G/A (Ala40Thr) polymorphism at p<0.05 in generalized vitiligo patients and control population.

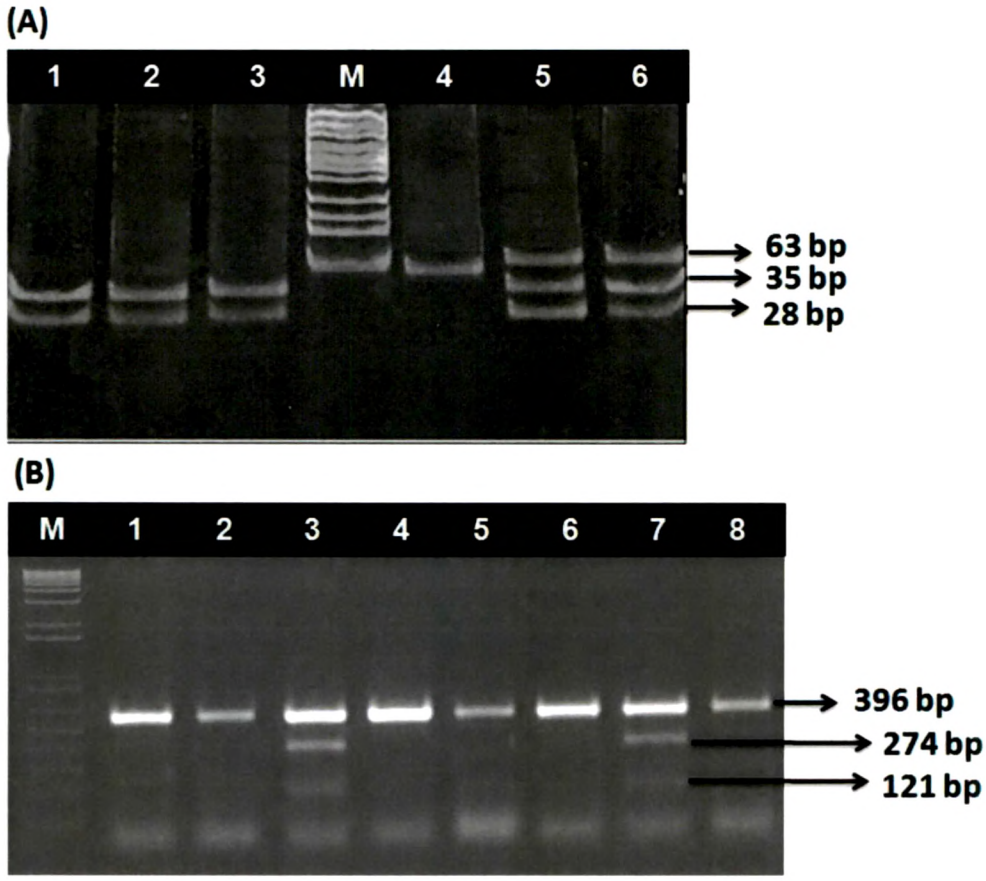


Figure 1. PCR-RFLP analysis of *SOD3* C/G (Arg213Gly) and G/A (Ala40Thr) polymorphism

(A) PCR-RFLP analysis of *SOD3* C/G (Arg213Gly) polymorphism on 20% polyacrylamide gel electrophoresis: lanes: 1, 2 & 3 show homozygous (CC) genotypes; lane: 5 shows homozygous (GG) genotype; lanes: 6 & 7 show heterozygous (CG) genotypes. lane: 4 shows a 50 bp ladder. (B) PCR-RFLP analysis of *SOD3* G/A (Ala40Thr) polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 2, 4, 5, 6 & 8 show homozygous (AA) genotypes; lanes: 3 & 7 show heterozygous (GA) genotypes; lane M shows 50 bp DNA ladder.

Table 3. Association studies for *SOD3* gene C/G (Arg213Gly) and G/A (Ala40Thr) 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)
rs1799895 (C/G; Arg213Gly)	Genotype	(n = 455)	(n = 548)			
	CC	170 (0.37)	245 (0.45)		0.881	
	CG	215 (0.47)	256 (0.47)	0.001[a]	(P)	
	GG	70 (0.16)	47 (0.08)			
	Allele				0.081	1.363
	C	555 (0.61)	746 (0.68)	0.001[b]	(C)	(1.134-
	G	355 (0.39)	350 (0.32)			1.639)
rs2536512 (G/A; Ala40Thr)	Genotype	(n = 448)	(n = 550)			
	GG	268 (0.60)	328 (0.60)		0.070	
	GA	148 (0.33)	188 (0.34)	0.802[a]	(P)	
	AA	32 (0.07)	34 (0.06)			
	Allele				0.315	0.9786
	G	684 (0.76)	844 (0.77)	0.873[b]	(C)	(0.7948-
	A	212 (0.24)	256 (0.23)			1.205)

'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 $p \leq 0.025$ due to Bonferroni's correction.

Table 4. Association studies of *SOD3* gene for C/G (Arg213Gly) and G/A (Ala40Thr) polymorphisms in generalized and localized vitiligo patients from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo Patients (Freq.)	Localized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
rs8192291 (C/G; Arg213Gly)	Genotype	(n = 310)	(n = 145)	(n = 548)			0.7378
	CC	119 (0.38)	51 (0.35)	245 (0.45)	0.003[a]	0.45 (GV)	(0.6008-0.9061) (GV)
	CG	141 (0.45)	74 (0.51)	256 (0.47)			
	GG	50 (0.17)	20 (0.14)	47 (0.08)	0.047[b]		
	Allele					0.402 (LV)	
	C	379 (0.61)	176(0.61)	746 (0.68)	0.004[c]		0.7243 (0.5542-0.9467) (LV)
	G	241 (0.39)	114(0.39)	350 (0.32)			
					0.021[d]	0.081 (C)	
rs2536512 (G/A; Ala40Thr)	Genotype	(n = 315)	(n = 133)	(n = 550)			0.9216
	GG	184 (0.58)	84 (0.63)	328 (0.60)	0.615[a]	0.086 (GV)	(0.7332-1.158) (GV)
	GA	106 (0.34)	42 (0.32)	188 (0.34)			
	AA	25 (0.08)	07 (0.05)	34 (0.06)	0.746[b]		
	Allele					0.564 (LV)	1.137 (0.8207-1.576) (LV)
	G	474 (0.75)	210(0.79)	844 (0.77)	0.482[c]		
	A	156 (0.25)	56 (0.21)	256 (0.23)			
					0.465[d]	0.315 (C)	

'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 $p \leq 0.025$ due to Bonferroni's correction.

Table 5. Association studies of *SOD3* gene for C/G (Arg213Gly) and G/A (Ala40Thr) 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 = 339)	(n = 116)	(n = 548)			0.7713 [a]
	CC	122 (0.36)	48 (0.41)	245(0.45)	0.194[a]	0.616	(0.5650-
rs8192291	CG	159 (0.47)	56 (0.48)	256(0.47)	0.0002[b]	(AV)	1.053)
(C/G;	GG	58 (0.17)	12 (0.11)	47 (0.08)	0.731[c]		
Arg213Gly)	Allele					0.461	0.6875 [b]
	C	403 (0.59)	152(0.66)	746(0.68)	0.119 [a]	(SV)	(0.5634-
	G	275 (0.41)	80 (0.34)	350(0.32)	0.0002[b]		0.8390)
					0.487[c]	0.081	
						(C)	0.8914 [c]
							(0.6610-
							1.202)
	Genotype	(n = 335)	(n = 113)	(n = 550)			1.232 [a]
	GG	206 (0.61)	62 (0.55)	328(0.60)	0.462[a]	0.073	(0.8712-
	GA	106 (0.32)	42 (0.37)	188(0.34)	0.714[b]	(AV)	1.741)
rs2536512	AA	23 (0.07)	09 (0.08)	34 (0.06)	0.588[c]		
(G/A;	Allele					0.617	1.034 [b]
Ala40Thr)	G	518 (0.77)	166(0.73)	844(0.77)	0.241 [a]	(SV)	(0.8224-
	A	152 (0.33)	60 (0.27)	256(0.23)	0.816[b]		1.299)
					0.304[c]	0.315	
						(C)	0.8392 [c]
							(0.6053-
							1.164)

'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,

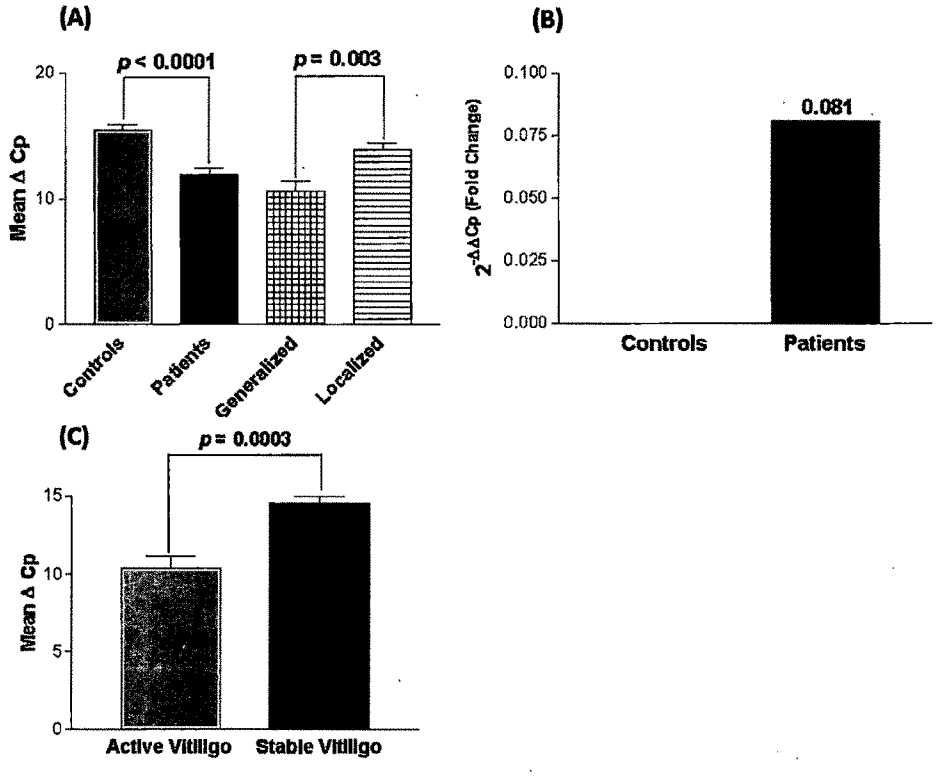
[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

4.3.3 Relative gene expression of *SOD3* in patients with vitiligo and controls:

Our *SOD3* gene expression studies showed significant increase in expression of *SOD3* transcripts in 166 vitiligo patients compared to 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values ($p<0.0001$) (Figure 2A). Moreover, generalized vitiligo patients showed significant higher expression of *SOD3* transcripts as compared to localized vitiligo patients ($p=0.003$) (Figure 2A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.081 fold change in the expression of *SOD3* transcript in patients as compared to controls (Figure 2B). In addition, we also checked the effect of *SOD3* expression on progression of the disease i.e. active and stable cases of vitiligo (Figure 2C). Interestingly, active vitiligo patients showed significant increase in expression of *SOD3* transcripts as compared to the patients with stable vitiligo ($p=0.0003$) suggesting the involvement of *SOD3* in disease progression.



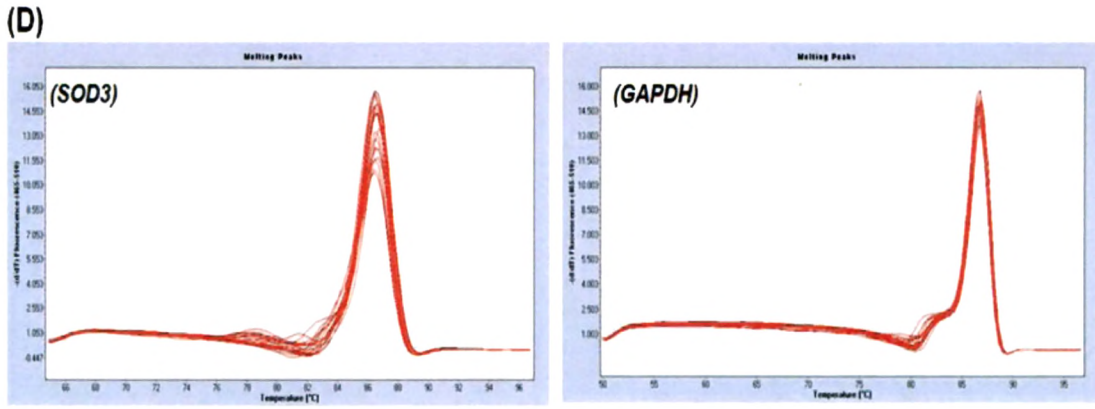


Figure 2. Relative gene expression of *SOD3* in controls and vitiligo patients:

(A) Expression of *SOD3* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significant increase in mRNA levels of *SOD3* as compared to controls (Mean ΔCp ± SEM: 11.93 ± 0.5535 vs 15.55 ± 0.3914; $p<0.0001$). Generalized vitiligo patients showed significant increase in mRNA levels of *SOD3* as compared to localized patients (Mean ΔCp ± SEM: 10.64 ± 0.7918 vs 13.92 ± 0.5248; $p=0.003$).

(B) Expression fold change of *SOD3* transcripts in 166 vitiligo patients against 175 controls showed 0.081 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *SOD3* 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 showed significant increase in mRNA levels of *SOD3* as compared to stable vitiligo patients (Mean ΔCp ± SEM: 10.42 ± 0.7306 vs 14.50 ± 0.4859; $p=0.0003$).

(D) Melt curve analysis of *SOD3* and *GAPDH* showing specific amplification.

4.3.4 Estimation of SOD3 activity in vitiligo patients and controls:

We have also analyzed the SOD3 specific activity in plasma samples of 482 vitiligo patients and 564 controls. SOD3 activity differed significantly between vitiligo patients and controls ($p<0.0001$) (Figure 3A). In particular, vitiligo patients showed 2 fold higher SOD3 activity as compared to controls. Also, patients with generalized

vitiligo showed significantly higher activity of SOD3 as compared to localized vitiligo (p=0.025) (Figure 3A).

Analysis based on the stage of progression of vitiligo revealed that active vitiligo patients had significant increase in SOD3 activity as compared to the patients with stable vitiligo (p=0.026) which suggests the involvement of SOD3 in disease progression (Figure 3B).

4.3.5 Correlation of *SOD3* C/G (Arg213Gly) and G/A (Ala40Thr) genotypes with SOD3 activity in vitiligo patients:

Further, the SOD3 activity was correlated with the genotypes obtained for Arg213Gly and Ala40Thr polymorphisms (Figure 3). Interestingly, SOD3 activity was significantly increased in patients with susceptible Arg213Gly GG genotypes and heterozygous CG genotypes as compared to controls (p=0.022; p=0.041) suggesting the involvement of Arg213Gly 'G' allele in increased activity of SOD3. However, no significant difference was observed in SOD3 activity in patients with CC genotypes as compared to controls (p=0.651) (Figure 3C). Moreover, there was no significant difference in SOD3 activity in patients with Ala40Thr GG, GA and AA genotypes as compared to controls (p=0.866; p=0.202; p=0.071) (Figure 3D) suggesting that Ala40Thr G/A polymorphism is not involved in increased SOD3 activity in patients.

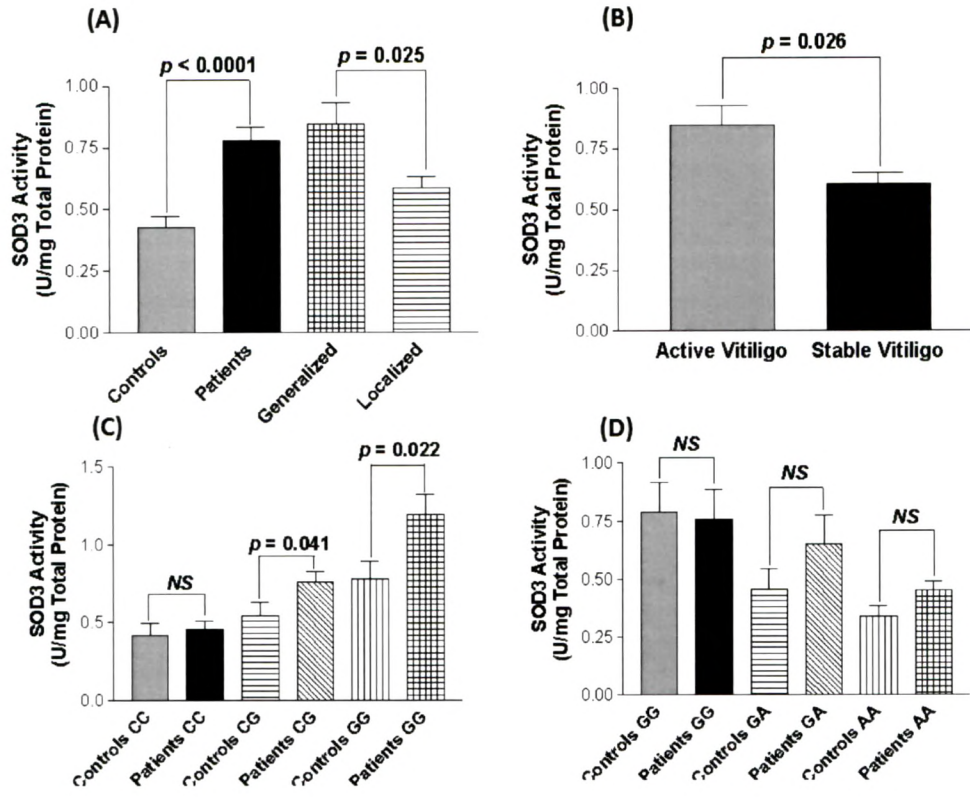


Figure 3. SOD3 activity in controls and vitiligo patients:

(A) SOD3 specific activity in 482 vitiligo patients and 564 controls. Vitiligo patients showed significant increase in SOD3 activity as compared to controls (Mean ± SEM: 0.7785 ± 0.05568 vs 0.4255 ± 0.04589; $p<0.0001$). Generalized vitiligo patients (n=328) showed significant increase in SOD3 activity as compared to localized patients (n=154) (Mean ± SEM: 0.8448 ± 0.08551 vs 0.5881 ± 0.04563; $p=0.025$).

(B) SOD3 specific activity with respect to progression of the disease in 352 patients with active vitiligo and 130 patients with stable vitiligo. Active vitiligo patients showed significant increase in SOD3 activity as compared to stable vitiligo patients (Mean ± SEM: 0.8490 ± 0.08076 vs 0.6040 ± 0.04752; $p=0.026$).

(C) SOD3 specific activity with respect to *SOD3* C/G (Arg213Gly) polymorphism in 455 vitiligo patients and 548 controls. Vitiligo patients showed significant increase in SOD3 activity with GG (Mean ± SEM: 1.194 ± 0.1316 vs 0.7793 ± 0.1126; $p=0.022$) and CG (Mean ± SEM: 0.7644 ± 0.06393 vs 0.5451 ± 0.08456; $p=0.041$) genotypes as compared to controls. There was no significant difference in the activity of SOD3 with CC genotypes (Mean ± SEM: 0.4592 ± 0.05117 vs 0.4179 ± 0.07670; $p=0.651$) as compared to controls.

(D) SOD3 specific activity with respect to *SOD3* G/A (Ala40Thr) polymorphism in 448 vitiligo patients and 550 controls. Vitiligo patients did not show significant difference in SOD3 activity with GG (Mean ± SEM: 0.7588 ± 0.1256 vs 0.7896 ± 0.1276; p=0.866), GA (Mean ± SEM: 0.6506 ± 0.1241 vs 0.4556 ± 0.08755; p=0.202) and AA genotypes (Mean ± SEM: 0.4525 ± 0.03683 vs 0.3391 ± 0.04543; p=0.071) as compared to controls. [*NS* = non-significant]

4.4 DISCUSSION

Vitiligo susceptibility is a complex genetic trait that may involve genes important for melanin biosynthesis, response to oxidative stress and regulation for autoimmunity as well as environmental factors. 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 a.l*, 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.

Superoxide dismutase scavenges the superoxide radicals and reduces its toxicity (Schallreuter *et al.*, 1991). SOD3 is found predominantly in the extracellular matrix of mammalian tissues (Marklund, 1984; Oury *et al.*, 1994). The C-terminal region is highly rich in basic amino acid residues (Hjalmarsson *et al.*, 1987). This polybasic region or the extracellular matrix (ECM)-binding region (Petersen *et al.*, 2004) is involved in its binding to heparin/heparan sulfate (Adachi *et al.*, 1992) and type I collagen (Petersen *et al.*, 2004). The heparin binding domain has also been proposed to act as a nuclear localization signal in certain cell types (Ookawara *et al.*, 2003), suggesting that SOD3 may provide antioxidant protection to DNA and nuclear

proteins. The C/G SNP present in *SOD3* gene which substitutes Arg to Gly at codon 213 inhibits ionic interaction between heparin and SOD3 which in turn results in 10 fold increase in SOD3 concentration in plasma (Sandstrom *et al.*, 1994). The Arg213Gly SNP located at the heparin-binding domain has been identified in a small proportion of the healthy population (Folz *et al.*, 1994) and has been associated with very high plasma SOD3 levels due to altered affinity of SOD3 for heparin at the endothelial cell surface (Sandstrom *et al.*, 1994; Adachi *et al.*, 1996; Karlsson *et al.*, 1994). The present study reports that C/G Arg213Gly polymorphism of *SOD3* is significantly associated with vitiligo susceptibility. In particular, the Arg213Gly 'G' allele is prevalent in patient group as compared to controls. Moreover, patients with active vitiligo had higher frequency of Arg213Gly 'G' allele as compared to controls suggesting that 'G' allele may be involved in progression of the disease. Interstingly, vitiligo patients showed approximately two fold higher activity of SOD3 as compared to controls. Also, generalized vitiligo patients showed increased SOD3 activity as compared to patients with localized vitiligo. The genotype-phenotype correlation further confirms the association of Agr213Gly 'G' allele with increased activity of SOD3 which can modulate the susceptibility of an individual towards vitliligo. Furthermore, patients with active vitiligo showed increased SOD3 activity as compared to stable vitiligo suggesting SOD3 involvement in the progression of the disease. The increased SOD3 activity may lead to increased H_2O_2 production which in turn result in substrate inhibition of the down stream antioxidant enzyme e.g. catalase ultimately leading to H_2O_2 mediated cell damage. Previously, Shajil and Begum (2006) reported low activity of catalse and glutathione peroxidase in vitiligo patients from Gujarat, supporting these results. We have also monitored *SOD3* expression in vitiligo patients and controls to know whether the increased activity of SOD3 is due to an increase in *SOD3* transcript levels. The expression results suggested that vitiligo patients had increased *SOD3* transcript levels as compared to controls. Also the expression of *SOD3* was higher in generalized vitiligo patients as compared to patients with localized vitiligo. Moreover, active vitiligo patients exhibited increased *SOD3* transcript levels as compared to stable vitiligo patients suggesting the possiblity that increased activity of SOD3 in patients may be due to increased expression of *SOD3*. Thus increased levels of SOD3 in vitiligo patients could enhance the systemic production of H_2O_2 which in turn results in increased oxidative stress in patients.

The second polymorphism G/A Ala40Thr addressed in this study is located in the amino terminal domain, which may affect the tetramerization of SOD3. The findings of Tamai *et al.* (2006) suggested the possibility that the Thr allele (Thr40Ala) is associated with impaired function of SOD3. However, the present study could not achieve significant association of this polymorphism with vitiligo susceptibility. Moreover, there was no significant difference in allele distribution of this polymorphism between different types of vitiligo i.e. active vs stable and generalized vs localized. In addtion, the genotype-phenotype correlation for this polymorphism remained uninformative as there was no significant difference in the SOD3 activity with different genotypes of Ala40Thr polymorphism in patients suggesting that this polymorphism may not be involved in increased activity of SOD3 as observed in vitiligo patients.

Interestingly, the present study suggested an increase in *SOD3* transcripts in vitiligo patients as compared to controls. This increased *SOD3* expression may be responsible for the increased SOD3 activity especially with respect to Arg213Gly polymorphism. Expression of *SOD3* can be induced by various factors. *In vitro* studies in skin fibroblasts showed that heparin and heparan sulfate could induce *SOD3* mRNA as well as protein expression. Moreover, in vascular smooth muscle cells (VSMC) and lung alveolar type 2 cells, inflammatory cytokines such as TNFα and IFNγ showed induction of *SOD3* mRNA and protein expression. While certain stimuli can also induce *SOD3* expression e.g. a variety of growth factors have been shown to repress *SOD3* mRNA expression, such as TGFβ in human fibroblasts (Marklund, 1992) and PDGF and FGF in vascular smooth muscle cells (Stralin & Marklund, 2001). Expression of the human *SOD3* is regulated by transcription factors such as Sp1 and Sp3 in the lung (Zelko *et al*, 2008). A putative NF-κB motif in the human *SOD3* promoter region has been proposed as the functional transcriptional binding site contributing to induction and coregulation of inducible nitric oxide synthase and SOD3 (Folz and Crapo, 1994; Fattman *et al.*, 2003, Brady *et al.*, 1997).

In conclusion, the present study signifies the imorptant role of SOD3 in pathogenesis and progression of vitiligo. In particular, Arg213Gly polymorphism of *SOD3* and increased levels of *SOD3* transcripts may in combination are responsible for

increased activity of SOD3 in extracelluar fluids resulting in increased H_2O_2 production. As the downstream antioxidant system in vitiligo patients was found to be disturbed, the H_2O_2 is not properly removed and may finally lead to oxidative damage to the melanocytes.

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