3.1 INTRODUCTION

Vitiligo is a common dermatological disorder characterized by expanding areas of hypo-pigmentation on the skin due to the selective destruction or inactivation of epidermal melanocytes (Taieb *et al*., 2007). It affects both genders equally and ~0.5-1% of the population in all ethnic groups worldwide (Taïeb and Picardo, 2009; Ezzedine *et al*., 2012). In India, the incidence rate of vitiligo is ~0.1 - 8.8% in India (Handa and Kaur, 1999; Sehgal and Srivastava, 2007). The mechanisms of melanocyte destruction have been widely debated including autoimmune, biochemical, neural, self-destructive, and genetic hypotheses (Ortonne and Bose, 1993; Castanet and Ortonne, 1997; Shajil *et al*., 2006; Mansuri *et al*., 2014a). Various findings have suggested that oxidative stress may be the triggering event of melanocyte degeneration in vitiligo (Passi *et al*., 1998; Jimbow *et al*., 2001; Laddha *et al*., 2013a, 2014). Oxidative stress is caused by an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage (Betteridge, 2000). Melanogenesis produces large amounts of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hence melanocytes are at risk of oxidative damage unless their antioxidant systems are functional (Nordlund and Ortonne, 1998; Hasse *et al*., 2004). There are convincing evidences for epidermal H_2O_2 accumulation and its association with low epidermal catalase levels in vitiligo (Schallreuter, 1991; Schallreuter *et al*., 1999; Rokos *et al*., 2002; Maresca *et al*., 2007 Sravani *et al*., 2009).

Catalase (CAT) is an endogenous antioxidant enzyme that protects cells against ROS damage by detoxifying H_2O_2 into H_2O and O_2 (Rohrdanz and Kahl, 1998). Nevertheless, the molecular basis of low catalase activity in vitiligo patients has not been determined. It has been suggested that genetic variations in the *CAT* gene may have deleterious effects on the expression or function of *CAT* (Goth *et al*., 2004). The human *CAT* gene is located on chromosome 11p13, consisting of 13 exons and 12 introns. The association of *CAT* gene polymorphisms and reduced catalase activity have been reported with other human diseases as well such as diabetes (Goth and Eaton, 2000), dyslipidaemia, catalasemia/hypocatalasemia (Goth, 2000), hypertension (Jiang *et al*., 2001), and

Alzheimer's disease (Goulas *et al*., 2002), including vitiligo (Casp *et al*., 2002; Liu *et al*., 2010).

Previously, the *CAT* exon 9 T/C (Asp389Asp; rs769217) polymorphism (silent substitution) was shown to be associated with vitiligo susceptibility in Caucasian and English populations (Casp *et al*., 2002; Gavalas *et al*., 2006). The study suggested that the variant genotype of *CAT* exon 10 C/T (Leu419Leu; rs11032709) SNP (also a silent substitution) could be associated with vitiligo susceptibility (Casp *et al*., 2002). Two other studies suggested that transcription of the *CAT* gene could be influenced by the polymorphisms located in its promoter region, resulting in low *CAT* expression (Go´th and Vitai, 1997; Park *et al*., 2006). Recently, a common polymorphism -262 G/A in the *CAT* promoter region has been found to be associated with altered catalase activity (Kodydková *et al*., 2014). Another, promoter polymorphism, *CAT* -89 A/T has been reported to be associated with vitiligo in Chinese population (Liu *et al*., 2010). However, our previous study on allelic association of *CAT* exon 9 T/C in vitiligo patients from Gujarat was shown to be uninformative (Shajil *et al*., 2007). Therefore, we hypothesized that other polymorphism/s might be responsible for the decreased catalase activity in vitiligo in Gujarat population.

Hence, in the present study we aimed 1) to assess the erythrocyte catalase activity and LPO levels, as well as *CAT* mRNA expression in skin and blood; 2) to investigate *CAT* 5'-UTR -20 T/C (rs1049982), promoter -89 A/T (rs7943316), -262 G/A (rs1001179) and exon 7 C/A (Ile242Ile; rs17886350); exon 10 C/T (Leu419Leu; rs11032709); exon 12 C/T (His492His; rs17880442); exon 13 G/A (Ala520Ala; rs35677492) polymorphisms and 3) to perform genotype-phenotype correlation analyses for these polymorphisms in vitiligo patients and controls from Gujarat.

3.2 MATERIALS AND METHODS

3.2.1 Study subjects

Both vitiligo patients and controls were of Gujarat origin (Table 1). Vitiligo patients had not received systemic immunosuppressive treatment or PUVA/UVB, for at least 1 month, and topical therapy for at least 2 weeks before skin biopsy. Four-millimeter punch biopsies were taken and snap frozen, from lesional and non-lesional skin of patients with

vitiligo (n= 12) and from non-inflamed, non-irritated skin of control individuals (n= 12). Further, we recruited 344 vitiligo patients and 497 controls for blood sample collection. The importance of the study was explained to all participants and written informed consent was obtained from all subjects before performing the studies. The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India and conducted according to the Declaration of Helsinki's principles.

Table 1: Demographic characteristics of vitiligo patients and unaffected controls recruited for skin and blood sample collection.

Particulars	Skin Samples		Blood Samples	
	Vitiligo Patients	Controls	Vitiligo Patients	Controls
	(n = 12)	(n = 12)	(n = 344)	(n = 497)
Average age (mean age ± SD)	29.38± 4.480 yr	28.33± 3.240 yr	31.22 ± 11.75 yr	30.41 ± 12.09 yr
Sex: Male	4	6	166 (48.26%)	246 (49.5%)
Female	8	6	178 (51.74%)	251 (50.5%)
Age of onset (mean age ± SD)	27.83 ± 6.05 yr	NA	22.07 ± 12.12 yr	NA
Duration of disease (mean ± SD)	6.83 ± 2.36 yr	NA	9.11 ± 6.64 yr	NA
Type of vitiligo				
Active vitiligo	7	NA	266 (77.33%)	NA
Stable vitiligo	5	NA	78 (22.67%)	NA
Localized vitiligo	4	NA	99 (28.78%)	NA
Generalized vitiligo	8	NA	245 (71.22%)	NA

3.2.2 Catalase (CAT) estimation:

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, USA). Catalase activity in the hemolysate was assayed by the method of Aebi (1984) in 344 vitiligo patients and 497 controls.

Hemolysate was normalized by Hb concentration. Briefly, decomposition of H_2O_2 by catalase was measured spectrophotometrically. Decrease in the absorbance was recorded at every 5 sec intervals for 15 seconds at 240nm. Catalase activity was expressed as U/gHb/s OR mmoles of H_2O_2 decomposed / g Hb/ sec. LPO levels were estimated according to the Beuge and Aust method (1978) as described earlier (Chapter II).

3.2.3 Genomic DNA Isolation and Genotyping of *CAT* polymorphisms

Genomic DNA was extracted from whole blood using QIAamp DNA Blood Kit (QIAGEN Inc., Valencia, USA), as described earlier (Chapter II). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype rs1001179, rs7943316, rs1049982, rs17880442 and rs35677492 SNPs whereas, amplification refractory mutation system (ARMS)-PCR method was used to genotype rs17886350 and rs11032709 SNPs. The primers used for genotyping are mentioned in Table 2 (Eurofins, Bangalore, India). PCR-RFLP and ARMS-PCR methods in detail are described earlier in 'Chapter II'.

3.2.4 RNA isolation and cDNA synthesis

Total RNA from skin biopsies and whole blood was extracted using RNA isolation kit (Ambion®, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA synthesis was performed using Verso cDNA Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions in the Mastercycler Gradient PCR (Eppendorf, Germany). Details are described in 'Chapter II'.

3.2.5 mRNA Expression

The transcripts levels of *CAT* in skin and in whole blood of vitiligo patients and controls were estimated by SYBR green method, using real-time PCR and 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 following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). Details are described in 'Chapter II'.

Table 2. Primers and restriction enzymes used for genotyping of *CAT* polymorphisms and expression.

Gene/ SNP	Nucleotide Substitution	Genomic Context	Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme	Digested Products (bp)
rs1001179	-262 G/A	Promoter	FP	AGAGCCTCGCCCCGCCGGACCG		167	*Sma*I	134+ 33
			RP	AGC TAT GGA GCG CAA GGC CCC ACC				
rs7943316	-89 A/T	Promoter	FP	AATCAGAAGGCAGTCCTCCC		250	*Hinf*I	177 + 73
			RP	TCGGGGAGCACAGAGTGTAC				
rs1049982	-20 T/C	5'UTR	FP	AGC CAA TCA GAA GGC AGT CCT C		166	*Pst*I	147+19
			RP	GCGTGCGGTTTGCTCTGC				
rs17886350	C/A	exon7	FP1	TTT TGT AGA CTG ACC AGG GCA TA	55	121	--	--
			FP2	TTT TGT AGA CTG ACC AGG GCA TC				
			RP	GTA CTT TCC TGT GGC AAT GGC				
rs11032709	C/T	Exon10	FP1	CCG GAA CAA CAG CCT TCT GCC C	59	302	--	--
			FP2	CCG GAA CAA CAG CCT TCT GCC T				
			RP	AGAA ACT GAG ACG GAG TCT GCA TG				
rs17880442	C/T	Exon12	FP	ACG TCT TTC CTC CCC TAT GG	53	402	BtsCI	253+148
			RP	CTG TCT TAA ATT GCC CAG CGC				
rs35677492	G/A	Exon13	FP	ACTGTTAGATTTCTTAGGCAGC	52	335	AciI	262+73
			RP	TTA CAC GGA TGA ACG CTA AGC				
hGH (PCR internal control)	-	-	FP	CCTTCCCAACCATTCCCTTA	61	428	--	--
			RP	TCACGGATTTCTGTTGTGTTTC				
CAT expression	-	-	FP	TAAGACTGACCAGGGCATC	63	201	--	--
			RP	CAAACCTTGGTGAGATCGAA				
GAPDH expression	-	-	FP	ATCCCATCACCATCTTCCAGGA	65	122	--	--
			RP	CAAATGAGCCCCAGCCTTCT				

'FP': forward primer; 'RP': reverse primer; 'bp': base pair; Underlined text: modified base to create the restriction enzyme recognition site.

3.2.6 Data analysis

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *CAT* 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 polymorphisms in different groups, considering the major genotype / allele as a reference group and were compared using chi-square test with 2×2 contingency tables using Prism 4 software (Graph Pad Software, USA, 2003). Odds ratio (OR) with 95% confidence interval (CI) for disease susceptibility was also calculated. Bonferroni's correction was applied for multiple testing of polymorphisms. Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi and He, 2005). The LD coefficients D' and r^2-values for the pair of most common alleles at each site were estimated using the Haploview programe version 4.1 (Barrett *et al*., 2005). Catalase activity, LPO levels, relative *CAT* mRNA expression were compared between different groups and genotype/ haplotype-phenotype correlation was analyzed and plotted by non-parametric unpaired t-test using Prism 4 software (Graph Pad Software, USA, 2003) to determine the statistical significance of data. *p*-values less than 0.05 were considered as statistically significant. Fold change in mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method. Statistical power of detection of association with the disease at 0.05 level of significance was determined by using G* Power software (Faul *et al*., 2007).

3.3 RESULTS

3.3.1 Genotyping of *CAT* promoter -89 A/T (rs7943316) and -262 G/A (rs1001179) polymorphisms:

The *CAT* promoter polymorphisms were addressed in 344 vitiligo patients and 497 controls where different genotypes were identified using PCR-RFLP (Figure 1). The genotype and allele frequencies differed significantly between patients and controls (p=0.005 and p=0.001 respectively; Table 3) for *CAT* -89 A/T polymorphism. In particular, the susceptible allele 'T' was more frequent in patients as compared to controls (p=0.001, OR=1.421, CI=1.152 to 1.753). 'TT' genotype was found to be significantly associated with vitiligo (p=0.008, OR=1.865, CI=1.170 to 2.971). Both

control and patient populations were under Hardy-Weinberg equilibrium (HWE) (p=0.635 and p=0.317 respectively) for *CAT* -89 A/T polymorphism.

Further, analysis based on disease progression revealed the increased frequency of susceptible genotype 'TT' and allele 'T' occurred predominantly in active vitiligo (AV) patients compared to controls (p=0.025 and p=0.002 respectively; Table 4). However, there was no significant difference between stable vitiligo (SV) patients vs. AV (p=0.789) and SV vs. controls (p=0.0972). Interestingly, analysis based on type of vitiligo showed higher frequency of 'TT' genotype and 'T' allele in generalized vitiligo (GV) patients as compared to controls (p=0.007 and p=0.0003 respectively; Table 4). However, there was no significant difference between localized vitiligo (LV) patients vs. GV (p=0.122) and LV vs. controls (p=0.349).

While, the genotype and allele frequencies did not differ between patients and controls (p=0.777 and p=0.530 respectively; Table 3) for *CAT* -262 G/A polymorphism. Also there was no significant difference in allele and genotype frequencies for this SNP with respect to type of vitiligo and disease progression (p>0.05; Table 4). Both control and patient populations were under HWE (p= 0.621 and p=0.375 respectively) for *CAT* -262 G/A polymorphism.

3.3.2 Genotyping of *CAT* 5'-UTR -20 T/C (rs1049982) polymorphism:

The *CAT* 5'-UTR T/C polymorphism was addressed in 344 vitiligo patients and 497 controls where different genotypes were identified using PCR-RFLP (Figure 1). The genotype and allele frequencies differed significantly between patients and controls (p<0.0001 and p<0.0001 respectively; Table 3). In particular, the minor allele 'C' was more frequent in patients as compared to controls (p<0.0001, OR=2.218, CI=1.626 to 3.025). 'TC' genotype was found to be significantly associated with vitiligo (p<0.0001, OR=2.453, CI=1.751 to 3.435). The control population was under HWE (p=0.213) whereas, patients were deviated (p=0.008) for *CAT* 5'-UTR polymorphism.

Further, analysis based on disease progression revealed the increased frequency of heterozygous 'TC' genotype occurred predominantly in AV patients compared to SV (p=0.014) and controls (p<0.0001; Table 4). Interestingly, the minor allele 'C' was predominant in AV patients compared to SV (p=0.018) and controls (p<0.0001) however,

there was no significant difference between SV patients and controls (p=0.424). Interestingly, analysis based on type of vitiligo showed higher frequency of 'TC' genotype in GV and 'LV patients compared to controls (p<0.0001 and p<0.0001 respectively; Table 4). The minor allele 'C' was predominant in GV as well as LV patients compared to controls (p<0.0001 and p<0.0001 respectively). However, there was no significant difference between GV and LV patients (p=0.171).

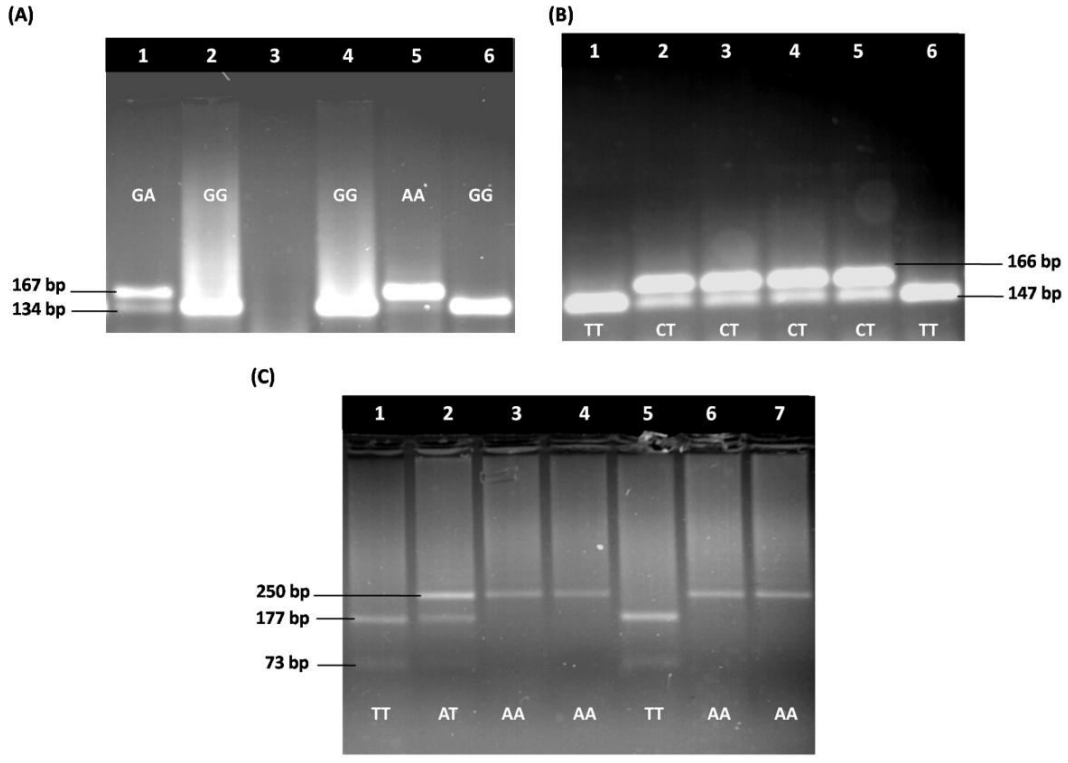

Figure 1. Genotyping of *CAT* gene polymorphisms: (A) PCR-RFLP analysis of *CAT* -262 G/A polymorphism on 3.5% agarose gel electrophoresis: lane 1 shows heterozygous GA; 2, 4 & 6 show homozygous GG and lane 5 shows homozygous AA genotype. **(B)** PCR-RFLP analysis of *CAT* -20 T/C polymorphism on 3.5% agarose gel electrophoresis: lane 1 & 6 show homozygous TT and lanes 2 to 5 show heterozygous TC genotype. **(C)** PCR-RFLP analysis of *CAT* -89 A/T polymorphism on 3.5% agarose gel electrophoresis: lane 1 & 5 show homozygous TT; lane 2 shows heterozygous AT and lanes 3,4,6 & 7 show homozygous AA genotype.

Table 3. Distribution of genotype and allele frequencies for *CAT* polymorphisms in Gujarat vitiligo patients and controls.

SNP	Genotype or Allele	Controls (n=497)	Patients (n=344)	*p* value[#] for Association	OR	95% CI
-262 promoter G/A (rs1001179)	GG	282 (0.57)	190 (0.55)	R	1	
	GA	182 (0.36)	127 (0.37)	0.814	1.021	0.8566 to 1.218
	AA	33 (0.07)	27 (0.08)	0.481	1.214	0.7070 to 2.086
	G	746 (0.75)	507 (0.74)	R	1	
	A	248 (0.25)	181 (0.26)	0.530	1.074	0.8597 to 1.341
-89 promoter A/T (rs7943316)	AA	68 (0.14)	32 (0.09)	R	1	
	AT	225 (0.45)	133 (0.39)	0.343	1.256	0.7837 to 2.013
	TT	204 (0.41)	179 (0.52)	0.008	1.865	1.170 to 2.971
	A	361 (0.36)	197 (0.99)	R	1	
	T	633 (0.64)	491 (0.71)	0.001	1.421	1.152 to 1.753
-20 5'UTR T/C (rs1049982)	TT	421 (0.85)	238 (0.69)	R	1	
	TC	75 (0.15)	104 (0.30)	<0.0001	2.453	1.751 to 3.435
	CC	1 (0.02)	2 (0.006)	0.272	3.538	0.3189 to 39.24
	T	917 (0.92)	580 (0.85)	R	1	
	C	77 (0.08)	108 (0.15)	<0.0001	2.218	1.626 to 3.025

n: number of subjects; R: reference group; OR: Odds Ratio; CI: Confidence Interval; [#]Vitiligo Patients vs. Controls using chi-squared test with 2 × 2 contingency table. Values are significant at $p \leq 0.017$ due to Bonferroni's correction.

Table 4. Distributions of genotype and allele frequencies of *CAT* polymorphisms in different subsets of vitiligo patients and controls.

SNP	Genotype or Allele	Controls (n=497)	AV (n=266)	SV (n=78)	*p* value	GV (n=245)	LV (n=99)	*p* value
-262 promoter G/A (rs1001179)	GG	282 (0.57)	145 (0.55)	45 (0.58)	R	134 (0.55)	56 (0.57)	R
	GA	182 (0.36)	99 (0.37)	28 (0.36)	0.734^{a} 0.727^{b} 0.888^{c}	93 (0.38)	34 (0.34)	0.601^{x} 0.660^{y} 0.797^{z}
	AA	33 (0.07)	22 (0.08)	5 (0.06)	0.551^{a} 0.375^{b} 0.918^{c}	18 (0.07)	9 (0.09)	0.682^{x} 0.658^{y} 0.430^{z}
	G	746 (0.75)	389 (0.73)	118 (0.76)	R	361 (0.74)	146 (0.74)	R
	A	248 (0.25)	143 (0.27)	38 (0.24)	0.530^{a} 0.411^{b} 0.874^{c}	129 (0.26)	52 (0.26)	0.986^{x} 0.567^{y} 0.698^{z}
-89 promoter A/T (rs7943316)	AA	68 (0.14)	27 (0.1)	5 (0.06)	R	22 (0.09)	10 (0.10)	R
	AT	225 (0.45)	97 (0.37)	36 (0.46)	0.179^{a} 0.7506^{b} 0.110^{c}	88 (0.36)	45 (0.46)	0.781^{x} 0.491^{y} 0.412^{z}
	TT	204 (0.41)	142 (0.53)	37 (0.47)	0.510^{a} 0.025^{b} 0.062^{c}	135 (0.55)	44 (0.44)	0.426^{x} 0.007^{y} 0.308^{z}
	A	361 (0.36)	151 (0.28)	46 (0.29)	R	132 (0.27)	65 (0.33)	R
	T	633 (0.64)	381 (0.72)	110 (0.71)	0.789^{a} 0.002^{b} 0.097^{c}	358 (0.73)	133 (0.67)	0.122^{x} 0.0003^{y} 0.349^{z}
-20 5'UTR T/C (rs1049982)	TT	421 (0.85)	175 (0.66)	63 (0.81)	R	176 (0.72)	62 (0.63)	R
	TC	75 (0.15)	89 (0.33)	15 (0.19)	0.015^{a} $<0.0001^{b}$ 0.353^{c}	67 (0.27)	37 (0.37)	0.074^{x} $<0.0001^{y}$ $<0.0001^{z}$
	CC	1 (0.02)	2 (0.007)	0 (0.0)	0.397^{a} 0.158^{b} 0.699^{c}	2 (0.01)	0 (0.0)	0.402^{x} 0.161^{y} 0.701^{z}
	T	917 (0.92)	439 (0.83)	141 (0.9)	R	419 (0.86)	161 (0.81)	R
	C	77 (0.08)	93 (0.17)	15 (0.1)	0.018^{a} $<0.0001^{b}$ 0.424^{c}	71 (0.14)	37 (0.19)	0.171^{x} $<0.0001^{y}$ $<0.0001^{z}$

n: number of subjects; R: reference group; AV: Active Vitiligo; SV: Stable Vitiligo; GV: Generalized vitiligo; LV: Localized vitiligo; [a]Active Vitiligo vs. Stable Vitiligo; [b]Active Vitiligo vs. Controls; [c]Stable Vitiligo vs. Controls; [x]Generalized vitiligo vs. Localized vitiligo; [y]Generalized vitiligo vs. Controls; [z]Localized vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table. Values are significant at $p \leq 0.017$ due to Bonferroni's correction.

3.3.3 Genotyping of *CAT* exon polymorphisms:

The genotyping results demonstrated that all the exon polymorphisms addressed of *CAT* (rs17886350, rs17880442, rs35677492 and rs11032709) showed only single genotype or allele in both patient and control groups (data not shown).

3.3.4 Linkage disequilibrium (LD) and haplotype analyses:

LD and haplotype analyses were performed with respect to *CAT* promoter -262 G/A (rs1001179); -89 A/T (rs7943316) and 5'-UTR -20 T/C (rs1049982) polymorphisms (Table3; Figure 2). The LD analysis revealed that the both promoter polymorphisms (rs1001179 and rs7943316) were in strong LD association (D'=0.847, r^2= 0.122). Whereas, 5'-UTR polymorphisms (rs1049982) was in low LD association with both promoter polymorphisms: rs1001179 (D'=0.015, r^2=0.000) and rs7943316 (D'=0.073, r^2=0.001). A haplotype evaluation of three polymorphic sites was performed and estimated frequencies of the haplotypes differed significantly between patients and controls (global *p*-value= $8.42e^{-005}$; Table 5). Interestingly, the frequency of susceptible haplotype '$A_{-262}T_{-89}C_{-20}$' containing the susceptible alleles of all three polymorphisms was significantly higher in patients compared to controls (p=0.0001) and increased the risk of vitiligo by 6.4-fold (OR=6.416; 95% CI= 2.183~18.854; Table 5). The '$G_{-262}A_{-89}C_{-20}$' haplotype was also pre-dominant in vitiligo patients as compared to controls (p=0.009). The wild type haplotype '$G_{-262}A_{-89}T_{-20}$' was more frequently observed in control group as compared to the patient (p=0.020, Table 5).

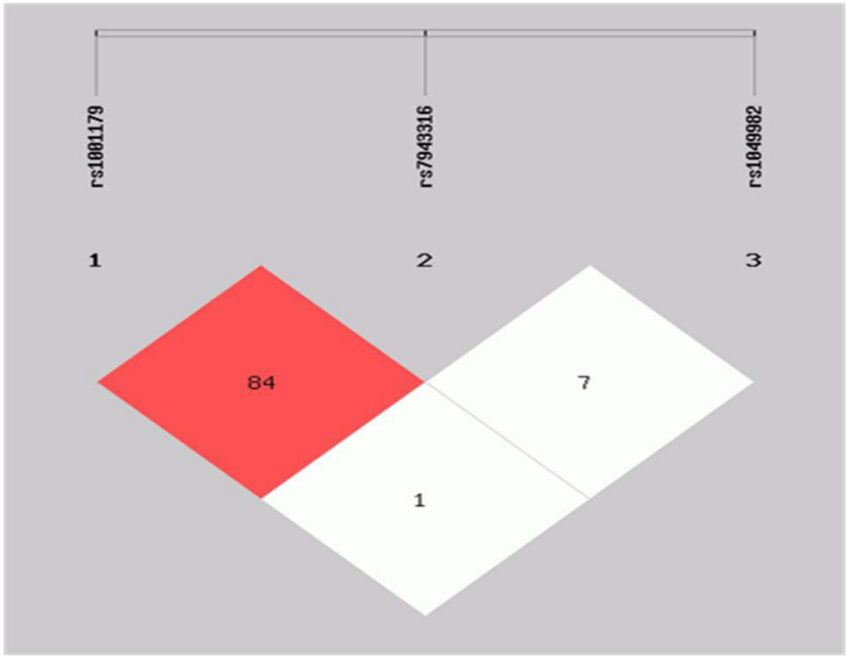

Figure 2. Linkage disequilibrium (LD) block: LD block with respect to *CAT* promoter -262 G/A (rs1001179); -89 A/T (rs7943316) and 5'-UTR -20 T/C (rs1049982) polymorphisms in Gujarat population.

Table 5. Distribution of haplotype frequencies for *CAT* promoter -262 G/A (rs1001179); -89 A/T (rs7943316); 5'-UTR -20 T/C (rs1049982) polymorphisms in Gujarat vitiligo patients and controls.

Haplotype	Patients (Freq.)	Controls (Freq.)	χ^2 value	*p* value	Odds Ratio [95% CI]
$\mathbf{A_{-262}\,A_{-89}\,T_{-20}}$	3.85 (0.013)	5.50 (0.011)	-	-	-
$\mathbf{A_{-262}\,T_{-89}\,C_{-20}}$	15.58 (0.051)	4.29 (0.008)	14.838	0.0001	6.416 [2.183~18.854]
$\mathbf{A_{-262}\,T_{-89}\,T_{-20}}$	68.57 (0.223)	117.20 (0.225)	0.006	0.938	0.987 [0.703~1.384]
$\mathbf{G_{-262}\,A_{-89}\,C_{-20}}$	20.86 (0.068)	15.37 (0.030)	6.769	0.009	2.391 [1.217~4.694]
$\mathbf{G_{-262}\,A_{-89}\,T_{-20}}$	74.29 (0.241)	165.12 (0.318)	5.434	0.020	0.684 [0.496~0.942]
$\mathbf{G_{-262}\,T_{-89}\,C_{-20}}$	13.56 (0.044)	15.33 (0.029)	1.227	0.268	1.519 [0.721~3.200]
$\mathbf{G_{-262}\,T_{-89}\,T_{-20}}$	111.29 (0.361)	197.17 (0.379)	0.244	0.621	0.929 [0.693~1.245]

'CI' represents Confidence Interval. (Haplotype frequency <0.03 in has been dropped and was ignored in analysis by the software).

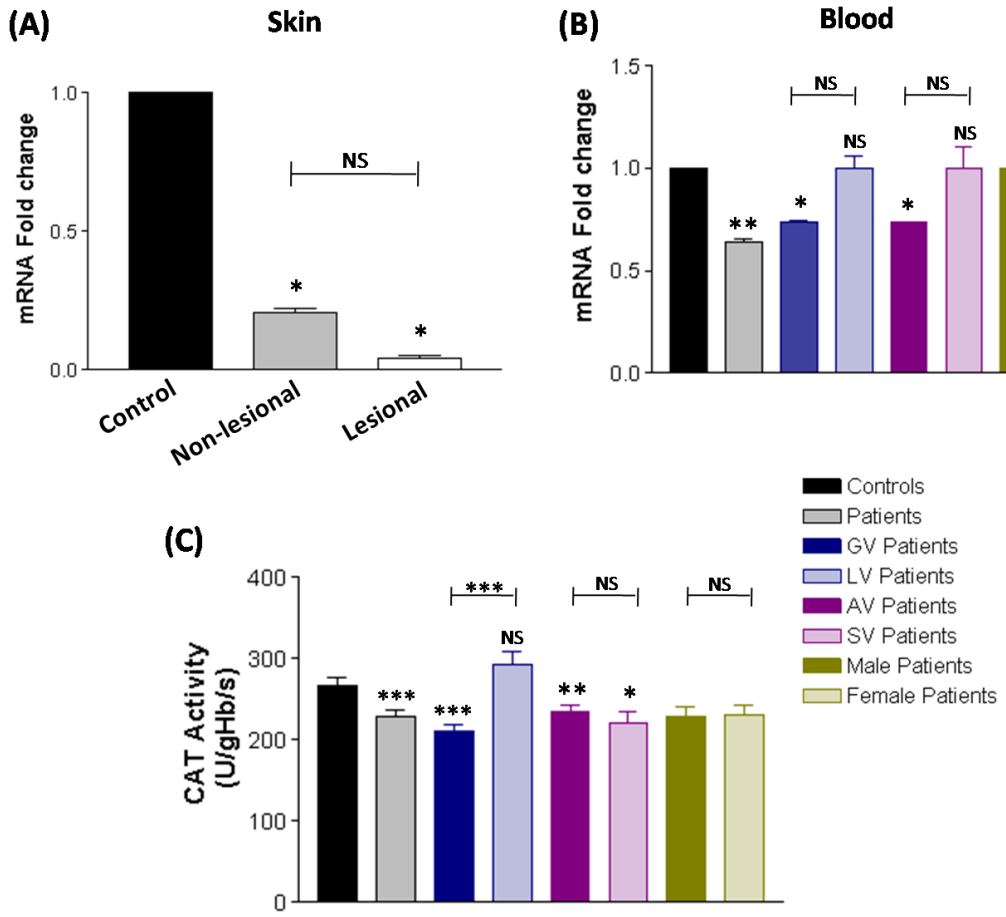

Figure 3: Catalase mRNA levels and activity in vitiligo patients. (A) Skin expression: Lesional and non-lesional skin of vitiligo patients showed significantly decreased *CAT* expression as compared to control skin (p=0.014 and p=0.039 respectively). No difference was observed for lesional vs. non-lesional skin (p=0.098). **(B) Blood expression:** Vitiligo patients showed significantly decreased *CAT* expression in whole blood, as compared to controls (p=0.001). GV and AV patients showed significantly decreased *CAT* expression, as compared to controls (p=0.043 and p=0.037 respectively). No difference was observed for control vs. LV (p=0.997); control vs. SV (p=0.986); LV vs. GV (p=0.120); SV vs. AV (p=0.311) and male vs. female patients (p=0.5179). **(C) Erythrocyte catalase activity:** Vitiligo patients showed significantly decreased catalase activity, as compared to controls (p=0.0007). GV, AV and SV patients showed significantly decreased catalase activity, as compared to controls (p<0.0001, p=0.009 and p=0.018 respectively). GV patients showed significantly decreased catalase activity, as compared to LV (p<0.0001). No difference was observed for AV vs. SV (p=0.404) and male vs. female patients (p=0.902). [AV: Active Vitiligo; SV: Stable Vitiligo; GV: Generalized vitiligo; LV: Localized vitiligo; *p<0.05; **p<0.01; ***p<0.001; NS: non-significant (p>0.05)].

3.3.5 *CAT* expression in skin of vitiligo patients:

The transcripts levels of *CAT* in skin of vitiligo patients (n=12) and controls (n=12) were estimated using real-time PCR by SYBR green method. Lesional (Mean ΔCt ± SEM: 12.79±1.137) as well as non-lesional skin (Mean ΔCt ± SEM: 10.48±0.7197) of patients showed significantly decreased expression of *CAT* as compared to control skin of healthy individuals (Mean ΔCt ±SEM: 8.209±0.5639) (p=0.014 and p=0.039 respectively; Figure 3A). However, there was no significant difference in *CAT* expression between lesional and non-lesional skin of patients (p= 0.098; Figure 3A).

3.3.6 *CAT* mRNA expression in blood of vitiligo patients:

Vitiligo patients (n=95) showed significantly decreased mRNA expression of *CAT* in blood as compared to controls (n=105) (Mean ΔCt ± SEM: 3.532±0.1109 vs. 2.899±0.1425; p=0.001; Figure 3B), as determined by mean ΔCt. Further, analysis based on type of vitiligo showed that GV patients (Mean ΔCt ± SEM: 3.335±0.129) exhibit significantly decreased *CAT* mRNA expression as compared to controls (p=0.043). However, there was no significant difference in the mRNA expression between LV patients (Mean ΔCt ± SEM: 2.898±0.2804) and controls (p=0.997), as well as between GV and LV patients (p=0.120; Figure 3B). In addition, analysis based on disease progression demonstrated that AV patients (Mean ΔCt ± SEM: 3.341±0.1422) showed significantly decreased expression of *CAT* mRNA as compared to controls (p=0.0368; Figure 3B). However, there was no significant difference in expression of *CAT* mRNA between SV patients (Mean ΔCt ± SEM: 2.908 ± 0.3949) and controls (p=0.9863), as well as between AV and SV patients (p=0.3106; Figure 3B). Moreover, analysis based on gender showed no difference in *CAT* mRNA expression between male and female patients (Mean ΔCt ± SEM: 3.053±0.1906 vs. 2.853±0.2445; p=0.518; Figure 3B).

3.3.7 Erythrocyte catalase activity and LPO levels in vitiligo patients:

Vitiligo patients showed significantly decreased catalase activity as compared to controls (Mean ± SEM: 229.2 ± 7.245 vs. 267.9 ± 8.686; p=0.0007; Figure 3C). Further, analysis based on type of vitiligo showed that GV (Mean ± SEM: 210.9 ± 7.842) showed significantly decreased catalase activity as compared to controls (p<0.0001) and LV patients (Mean ± SEM: 293.2 ± 15.63) (p<0.0001). However, there was no significant

difference in catalase activity between LV patients and controls (p=0.212). Furthermore, analysis based on disease progression demonstrated that both AV patients (Mean ± SEM: 235.1 ± 8.433) as well as SV patients (Mean ± SEM: 220.8 ± 14.22) exhibit significantly decreased catalase activity as compared to controls (p=0.009 and p=0.018 respectively; Figure 3C). However, there was no significant difference in catalase activity between AV and SV patients (p=0.404). Moreover, analysis based on gender showed no difference in catalase activity between male and female patients (Mean±SEM: 229.8 ± 10.24 vs. 231.6 ± 10.57; p= 0.902; Figure 3C). In addition, vitiligo patients showed significantly higher lipid peroxidation (LPO) levels as compared to controls (p<0.0001; data not shown).

3.3.8 Genotype -phenotype correlation analysis for *CAT* polymorphisms:

CAT mRNA, catalase activity and LPO levels were analyzed with respect to *CAT* 5'-UTR -20 T/C; promoter -89 A/T and -262 G/A polymorphisms. Individuals with 'TC+CC' genotypes for 5'-UTR -20 T/C polymorphism showed significantly decreased *CAT* mRNA levels as compared to individuals with TT genotype (Mean ΔCt±SEM: 3.957 ± 0.2785 vs. 3.181 ± 0.2394; p=0.039; Figure 4A). Individuals with TT genotype for -89 A/T polymorphism showed significantly decreased *CAT* mRNA levels as compared to individuals with AA genotype (Mean ΔCt±SEM: 4.652 ± 0.3201 vs. 3.652 ± 0.2714; p=0.027; Figure 4A). However, there was no significant difference for *CAT* expression between individuals with AT (Mean ΔCt±SEM: 3.840 ± 0.2839) and AA genotype (p=0.648), also between AT and TT genotype (p=0.063). While, no significant difference was observed for *CAT* expression among individuals with GG (Mean ΔCt± SEM: 3.967 ± 0.2747), GA (Mean ΔCt±SEM: 3.815 ± 0.1943) and AA (Mean ΔCt± SEM: 3.494 ± 0.9800) genotypes for -262 G/A polymorphism (p>0.05; Figure 4A).

Consistent with the mRNA levels, individuals with 'TC+CC' genotypes for -20 T/C polymorphism showed significantly decreased catalase activity as compared to individuals with TT genotype (Mean ΔCt±SEM: 212.5 ± 12.47 vs. 256.8 ± 9.942; p=0.016; Figure 4B). Further, individuals with TT genotype (Mean ΔCt ± SEM: 198.9 ± 10.17) for -89 A/T polymorphism showed significantly decreased catalase activity as compared to individuals with AA (Mean ΔCt±SEM: 244.5 ± 23.09) as well as AT genotype (Mean ΔCt ± SEM: 232.6 ± 12.19) (p=0.041 and p=0.034 respectively; Figure 4B).

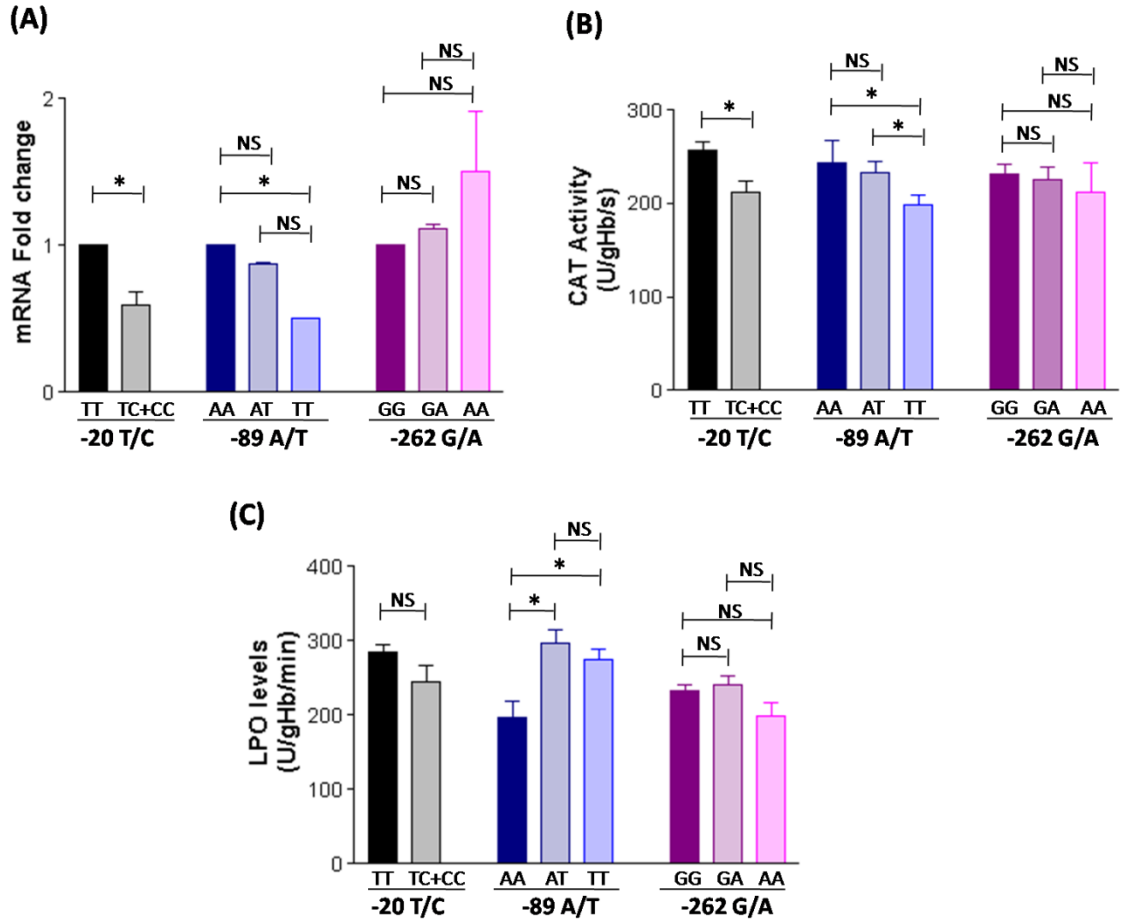

Figure 4: Genotype-phenotype correlation analysis for *CAT* 5'-UTR -20 T/C; promoter -89 A/T and -262 G/A polymorphisms with respect to: (A) *CAT* mRNA levels: Individuals with -20 'TC+CC' genotypes showed decreased *CAT* mRNA as compared to 'TT' genotype (p=0.039). Individuals with -89 'TT' genotype showed decreased *CAT* mRNA as compared to 'AA' genotype (p=0.027). No difference was observed among -262 G/A genotypes. **(B) Catalase activity:** Individuals with -20 'TC+CC' genotypes showed decreased catalase activity as compared to 'TT' genotype (p=0.016). Individuals with -89 'TT' genotype showed decreased catalase activity as compared to 'AA' and 'AT' genotype (p=0.041 and p=0.034 respectively). No difference was observed among -262 G/A genotypes. **(C) LPO levels:** Individuals with -89 'TT' and 'AT' genotypes showed increased LPO as compared to 'AA' genotype (p=0.023 and p=0.021 respectively). No difference was observed for -20 T/C and -262 G/A genotypes. [*p<0.05; NS: non-significant (p>0.05)].

However, there was no significant difference between AT and AA genotype (p=0.628). While, no significant difference was observed for catalase activity among individuals with GG (Mean ΔCt ± SEM: 232.3 ± 10.25), GA (Mean ΔCt ± SEM: 225.8 ± 13.16) and AA (Mean ΔCt ± SEM: 212.5 ± 30.95) genotypes for -262 G/A polymorphism (p>0.05; Figure 4B). Moreover, individuals with TT (Mean ΔCt ± SEM: 275.3 ± 13.54) as well as AT genotype (Mean ΔCt ± SEM: 297.4 ± 17.65) for -89 A/T polymorphism showed significantly higher LPO levels as compared to individuals with AA (Mean ΔCt ± SEM: 197.6 ± 20.87) genotype (p=0.023 and p=0.021 respectively; Figure 4C). However, there was no significant difference between AT and TT genotype (p=0.318). Also, no significant difference was observed in LPO levels among individuals with different genotypes *CAT* 5'-UTR -20 T/C; promoter -262 G/A polymorphisms (p>0.05; Figure 4C).

3.3.9 Haplotype-phenotype correlation analysis for *CAT* promoter -262 G/A; -89 A/T and 5'-UTR -20 T/C polymorphisms:

Catalase activity and LPO levels were analyzed with respect to haplotypes of *CAT* -262 G/A; -89 A/T and -20 T/C polymorphisms. Individuals with '$A_{-262}A_{-89}T_{-20}$' (Mean ΔCt ± SEM: 148.0 ± 21.48), '$A_{-262}T_{-89}T_{-20}$' (Mean ΔCt ± SEM: 157.8 ± 25.43) and '$A_{-262}T_{-89}C_{-20}$' (Mean ΔCt ± SEM: 188.4 ± 33.07) haplotypes showed significantly decreased catalase activity as compared to individuals with '$G_{-262}A_{-89}T_{-20}$' (Mean ΔCt ± SEM: 306.6 ± 36.85) haplotype (p=0.0128, p=0.002 and p=0.032 respectively; Figure 5A). Whereas, no significant difference in catalase activity was observed for '$G_{-262}A_{-89}C_{-20}$' (Mean ΔCt ± SEM: 220.2 ± 30.35), '$G_{-262}T_{-89}T_{-20}$' (Mean ΔCt ± SEM: 242.0 ± 19.89) and '$G_{-262}T_{-89}C_{-20}$' (Mean ΔCt ± SEM: 228.5 ± 34.11) haplotypes as compared to individuals with '$G_{-262}A_{-89}T_{-20}$' haplotype (p=0.121, p=0.101 and p=0.139 respectively).

Moreover**,** individuals with '$A_{-262}A_{-89}T_{-20}$' (Mean ΔCt ± SEM: 271.3 ± 36.12), '$A_{-262}T_{-89}T_{-20}$' (Mean ΔCt ± SEM: 234.9 ± 14.57) and '$A_{-262}T_{-89}C_{-20}$' (Mean ΔCt ± SEM: 254.5 ± 23.99) haplotypes showed significantly increased LPO as compared to individuals with '$G_{-262}A_{-89}T_{-20}$' (Mean ΔCt ± SEM: 192.4 ± 13.70) haplotype (p=0.025, p=0.038 and p=0.027 respectively; Figure 5B). While, no significant difference in LPO levels was observed for '$G_{-262}A_{-89}C_{-20}$' (Mean ΔCt ± SEM: 208.8 ± 22.05), '$G_{-262}T_{-89}T_{-20}$' (Mean

ΔCt ± SEM: 224.0 ± 19.92) and '$G_{-262}T_{-89}C_{-20}$' (Mean ΔCt ± SEM: 202.3 ± 18.39) haplotypes as compared to individuals with '$G_{-262}A_{-89}T_{-20}$' haplotype (p= 0.509, p= 0.186 and p= 0.691 respectively).

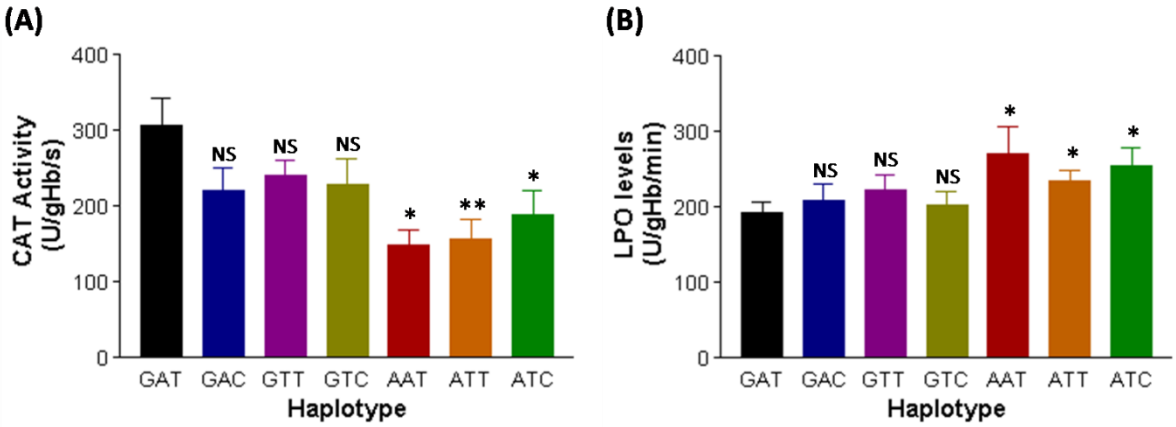

Figure 5: Haplotype-phenotype correlation analysis for *CAT* promoter -262 G/A; -89 A/T and 5'-UTR -20 T/C polymorphisms with respect to: (A) Catalase activity: Individuals with '$A_{-262}A_{-89}T_{-20}$', '$A_{-262}T_{-89}T_{-20}$' and '$A_{-262}T_{-89}C_{-20}$' haplotypes showed decreased catalase activity as compared to '$G_{-262}A_{-89}T_{-20}$' haplotype (p=0.013, p=0.002 and p=0.032 respectively). No difference was for '$G_{-262}A_{-89}C_{-20}$', '$G_{-262}T_{-89}T_{-20}$' and '$G_{-262}T_{-89}C_{-20}$' haplotypes (p=0.121, p=0.101 and p=0.139 respectively). **(B) LPO levels:** Individuals with '$A_{-262}A_{-89}T_{-20}$', '$A_{-262}T_{-89}T_{-20}$' and '$A_{-262}T_{-89}C_{-20}$' haplotypes showed increased LPO as compared to '$G_{-262}A_{-89}T_{-20}$' haplotype (p= 0.025, p=0.038 and p=0.027 respectively). No difference was for '$G_{-262}A_{-89}C_{-20}$', '$G_{-262}T_{-89}T_{-20}$' and '$G_{-262}T_{-89}C_{-20}$' haplotypes (p= 0.509, p= 0.186 and p= 0.691 respectively). [*p<0.05; **p<0.01; NS: non-significant (p>0.05)].

3.4 DISCUSSION

The auto-cytotoxic premise suggests that melanocyte destruction in vitiligo could be related initially to an increased oxidative stress, with subsequent accumulation of H_2O_2 in the epidermis of patients (Schallreuter *et al.*, 1999; Spencer *et al.*, 2007; Namazi, 2007). A diverse body of evidence, including several cellular abnormalities and biochemical defects that have been reported in vitiligo patients, support this hypothesis (Maresca *et*

al., 1997; Passi *et al*., 1998; Schallreuter, 1999; Rokos *et al*., 2002; Hasse *et al*., 2004). Alteration in the antioxidant system, such as the low catalase, glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase (G6PD) levels and high superoxide dismutase (SOD) levels have been demonstrated in the epidermis and/ blood of vitiligo patients (Schallreuter *et al*., 1991; Schallreuter *et al*., 1999; Yildirim *et al*., 2003; Agrawal *et al*., 2004; Hazneci *et al*., 2005; Shajil and Begum, 2006; Shajil *et al*., 2007; Laddha *et al*., 2013b; Agrawal *et al*., 2014; Mansuri *et al*., 2016). When additional oxidative events occur, the pro-oxidant systems outbalance the antioxidant, potentially producing oxidative damage to lipids, proteins, carbohydrates, and nucleic acids, ultimately leading to cell death in severe oxidative stress. Interestingly, reduced GPx and increased SOD levels were correlated with higher LPO levels in patients with vitiligo in our previous studies (Laddha *et al*., 2013b; Mansuri *et al*., 2016). Also, the associations of genetic variations in *GPX1*, *SOD2* and *SOD3* genes with vitiligo susceptibility have been shown in Gujarat population (Laddha *et al*., 2013; Mansuri *et al*., 2016). Previously, reduced levels of catalase in suction blister roofs taken from the lesional and non-lesional epidermis of vitiligo patients has been demonstrated (Schallreuter *et al*., 1991). A decrease in catalase activity is expected to increase the concentration of H_2O_2 in the epidermis of vitiligo patients. Decreased catalase activity and accumulation of H_2O_2 in the epidermis of vitiligo patients, resulting in more sensitivity of melanocytes to oxidative stress has also been reported (Schallreuter *et al*., 1991; Jimbow *et al*., 2001). In agreement to these reports, we observed decreased *CAT* mRNA levels in lesional and non-lesional skin as well as in blood of vitiligo patients, along with decreased erythrocyte catalase activity (Figure 3). In addition, patients with active and generalized vitiligo showed significantly lower catalase mRNA and activity compared to controls, indicating the important role of catalase in disease progression as well.

Catalase plays an important role against severe oxidative stress in the cells (Kinnula *et al*. 1992), and genetic variations in the *CAT* gene could have deleterious effects on the function or expression of CAT, which may give rise to high sensitivity to H_2O_2 (Go´th *et al*., 2004). In the present study, we investigated whether allelic variants of *CAT* contribute to the risk of developing vitiligo. In the case–control study, we demonstrated a statistically significant increased risk of vitiligo associated with the variant *CAT* -20 T/C

and -89 A/T genotypes, but no evident risk was associated with the -262 G/A or exonic variants (Table 3). Compared with *CAT* -20 'TC' heterozygotes, there seemed to be a disparity in the significance for homozygous 'CC' even with increased risk for disease (OR=3.538, CI=0.3189 - 39.24). We inferred that this might be due to less number of homozygotes (CC) observed (Table 3). In contrast, our study showed that *CAT* -89 'TT' genotype was associated with a significantly increased risk of generalized and active vitiligo, whereas -20 'TC' genotype was associated with generalized/ localized and active vitiligo (Table 4).

For 5'-UTR SNPs, allele variation could not be detected in any of the vitiligo patients/ control subjects in English population. On contrary, we found significant association of *CAT* 5'-UTR -20T/C polymorphism with susceptibility to vitiligo in Gujarat population (Table 3). Previously, the association of *CAT* -89A/T polymorphism with vitiligo risk has been reported in Chinese population (Liu *et al*., 2010). However, no association was observed between *CAT* -89A/T polymorphism and vitiligo susceptibility in the Korean (Park *et al*., 2006), Turkish (Akbas *et al*., 2013) and Egyptian (Younes *et al*., 2014) populations. The *CAT* -89A/T, -20T/C, polymorphisms were also associated with the risk of osteonecrosis of the femoral head in the Korean population (Kim *et al*., 2008). Controversial results regarding the association of *CAT* -89A/T polymorphism and vitiligo seems to be related with role of ethnicity. Our results are in accordance to Chinese population (Liu *et al*., 2010), suggesting *CAT* -89A/T variant as a common factor for vitiligo susceptibility in both the ethnicities.

No association was reported for *CAT* -262 G/A polymorphism with vitiligo in English (Gavalas *et al*., 2006) and Hungarian (Ko´sa *et al*., 2012) populations. Our results are in accordance to these reports, supporting no association between *CAT* -262 G/A allelic variants and vitiligo susceptibility. However, *CAT* -262G/A polymorphism has been associated with diabetic neuropathy (Chistiakov *et al*., 2006) and Crohn's disease (Iborra *et al*., 2013). D'souza *et al*. (2008) have reported that the *CAT* -262 G/A SNP was not associated with SLE whereas; the coding SNP's namely rs35677492, rs17880442, rs11032709, rs769217 and rs17886350 were non-informative. The 419C/T (rs11032709) polymorphism was not associated with vitiligo in Caucasian, English and Chinese

populations (Casp *et al*., 2002; Gavalas *et al*., 2006; Liu *et al*., 2010). Casp *et al*., (2002) have reported homozygous 'TT' genotype for *CAT* 419C/T in nearly all the samples analyzed in a North American population. Conversely, only homozygous 'CC' genotype was observed in Chinese population (Liu *et al*., 2010). Our results are in agreement to Chinese population, where the T allele could not be detected in any of the subjects, indicating that all individuals recruited in present study were homozygous for the C allele. In the Gujarat population, *CAT* rs1001179, rs17886350, rs11032709, rs17880442 and rs35677492 polymorphisms were not found to have influence on the risk of vitiligo.

The interaction of multiple polymorphisms within a haplotype can affect biological phenotype (Drysdale *et al*., 2000). The genotype and allele frequencies for *CAT* -89 A/T and exon 9 C/T SNPs did not differ significantly between vitiligo patients and controls in Korean population however; the haplotype of two polymorphisms was associated with vitiligo (Park *et al*., 2006). In the present study, although the *CAT* -262 G/A had no influence on the risk of vitiligo, we found that the *CAT* -262G/A, -89A/T and -20C/T variants may have a joint effect on the risk for vitiligo when the effects of these three polymorphisms were analyzed together in the context of their haplotype (Table 5). In the haplotype analysis, the '$A_{-262}T_{-89}C_{-20}$' haplotype with variant alleles was found to be associated with vitiligo risk of 6.4 fold (Table 5). Moreover, we observed a significant interaction between *CAT* -89A/T and *CAT* -262G/A polymorphisms, as suggested by strong LD association (Figure 2). Compared with the wild-type haplotype '$G_{-262}A_{-89}T_{-20}$', the haplotypes containing more variant alleles: '$A_{-262}T_{-89}C_{-20}$' (OR=6.416) and '$G_{-262}A_{-89}C_{-20}$' (OR=2.391) were associated with a higher risk of vitiligo in Gujarat population.

In addition to substrate inhibition of catalase activity due to high H_2O_2 levels in the epidermis of vitiligo patients, genetic variants in the *CAT* gene have detrimental effects on the expression or function of catalase (Forsberg *et al*., 2001; Go´th *et al*., 2004; Wood *et al*., 2008). Interestingly, our genotype-phenotype correlation analyses revealed that individuals with variant genotypes for *CAT* -89 A/T and -20 T/C polymorphisms exhibit low mRNA levels and reduced catalase activity as compared to their wild genotypes (Figure 4A & 2B), resulting into oxidative stress and increased risk of vitiligo. Moreover,

variant genotypes for *CAT* -89 A/T polymorphism demonstrated higher LPO levels as compared to wild genotype (Figure 4C), indicating its important role in oxidative damage. The variant promoter sequence region can bind different transcription factors, resulting in observed differences in promoter activity, which may provide a clue for future epidemiological association studies (Forsberg *et al*., 2001). The cis-regulatory variant -20 T/C (rs1049982) in 5'-UTR of *CAT* contributes to inter-individual variation in allele-specific *CAT* expression (Yeo *et al*., 2014). Wang *et al*. (2012) have shown that microRNA miR-147b interacts with the 5'-UTR of mRNA encoding catalase and can regulate *CAT* allelic expression imbalance through the SNP in the 5'-UTR. However, our previous study on skin miRNA signatures in vitiligo patients showed no difference in miR-147b expression (Mansuri *et al*., 2014b & c). The miR-147b interacts with the *CAT* 5'-UTR rs1049982 indeed affects regulation of *CAT* expression by *in vitro* where, miR-147b had a higher binding affinity for the 'C' allele than for the 'T' allele (Wang *et al*., 2012). Taken together, *CAT* -20T/C (rs1049982) is a unique SNP that resides in a miRNA gene regulatory loop (Wang *et al*., 2012) which regulate the *CAT* expression in vitiligo patients, demonstrating its crucial role in vitiligo susceptibility.

Genotype-dependent response of catalase activity to oxidative stress might be related to the predisposition of *CAT* variant allele carriers to the oxidative stress mediated disorders (Komina, *et al*., 2012). The *CAT* -262 G/A polymorphism influence *CAT* expression at the transcriptional level (Forsberg *et al*., 2001). The human *CAT* promoter is GC-rich, has several putative Sp1 binding sites and lacks a TATA box (Quan *et al*., 1986). Besides, studies are necessary to assess potential transcriptional mechanisms or transcriptional factor binding site for *CAT* -89A/T loci. Whereas, comparison of the 'GG' genotype and the 'AA' genotype for *CAT* -262T/C SNP in the vitiligo patients showed a non-significant increase in blood catalase activity in Hungarian population (Kosa *et al*., 2012). In the present study, the 'AA' genotype which results in lower *CAT* expression did not occur more frequently in vitiligo patients compared to controls (Table 3), suggesting that this polymorphism is not associated with vitiligo susceptibility. It has been demonstrated that peripheral blood mononuclear cells (PBMCs) from individuals with 'GA' and 'AA' genotypes for *CAT* -262T/C SNP had decreased catalase activity when exposed to H_2O_2 (Komina, *et al*., 2012; Bastaki *et al*., 2006; Ahn *et al*. 2005). Based upon the evidences it

could be hypothesized that substrate H_2O_2 can considerably affect the catalase enzyme activity resulting in inactivation of catalase (Gibbons *et al.* 2006). Consequently, a decreased *CAT* expression in individuals with variant allele/s may lead to further diminishing the catalase activity (Komina, *et al*., 2012). In addition, the haplotype-phenotype correlation analysis revealed that individuals with susceptible haplotypes: '$A_{-262}T_{-89}C_{-20}$', '$A_{-262}A_{-89}T_{-20}$' and '$A_{-262}T_{-89}T_{-20}$' showed significantly lower catalase activity and higher LPO levels as compared to individuals with wild haplotype '$G_{-262}A_{-89}T_{-20}$' (Figure 5). Therefore, individuals with lower *CAT* mRNA or activity and the *CAT* -89 A/T and -20 T/C variant genotypes/ haplotype might have increased risk for developing vitiligo compared with those with normal catalase activity and wild genotypes/ haplotype. Low *CAT* mRNA/ activity and the *CAT* -262 'A', -89 'T' and -20 'C' alleles indicate susceptibility to oxidative damage in the Gujarat population. Because of uncontrolled biases in the selection of subjects and limited sample size, larger and multi-population-based studies with inclusion of more SNPs in genes involved in oxidative stress are warranted to confirm these findings.

Treatment for vitiligo is also always very challenging. Most current therapies require extended treatment plans that may last many months to years and may still result in disappointing outcomes (Taieb and Picardo, 2009). This lack of treatment success indicates that in addition to present clinical selection criteria, the suitable biomarkers for vitiligo diagnosis, monitoring harmful immune events and predicting vitiligo progression and therapeutic response, would be most helpful. Low catalase, reduced glutathione peroxidase and increased monoamine oxidase A activities, perturbed (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin ($6BH_4$) recycling and "oxygen burst" via NADPH oxidase from a cellular infiltrate, are the potential sources for H_2O_2 generation in epidermis of vitiligo patients (Schallreuter *et al*., 1991; Schallreuter *et al*., 1999). H_2O_2 oxidizes $6BH_4$ to 6-biopterin, which is cytotoxic to melanocytes *in vitro*. Apart from inactivation of catalase, H_2O_2 accumulation can also lead to vacuolation in epidermal melanocytes and keratinocytes. Vacuolation was also observed in melanocytes *in vitro,* established from lesional and non-lesional skin of vitiligo patients, which was reversed upon addition of catalase (Schallreuter *et al*., 1999). The authors substituted the impaired catalase with a "pseudocatalase". Pseudocatalase is a bis-manganese III-EDTA-$(HCO_3\text{-})_2$ complex

activated by UVB or natural sun. Low-dose, narrow-band UV-B-activated pseudocatalase PC-KUS has also been used in a pilot study on 71 children patients, showing remarkable repigmentation even in long lasting disease which is an effective treatment for childhood vitiligo (Schallreuter *et al*., 2008).

In conclusion, this is the first report suggesting that *CAT* promoter and 5'-UTR polymorphisms may decrease *CAT* mRNA expression and activity and affect the risk of vitiligo in the Gujarat population. The *CAT* -89A/T and -20T/C variant genotypes were associated with susceptibility to vitiligo and had interactions with -262G/A polymorphism in promoter. The Genotype-/ haplotype-phenotype correlation showed a relationship between increased risk and decreased *CAT* mRNA/ activity as well as increased LPO levels.

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