CHAPTER IV A

ASSOCIATION OF *CATALASE* T/C EXON 9 POLYMORPHISM IN RELATION TO CATALASE ACTIVITY AND OXIDATIVE STRESS WITH VITILIGO SUSCEPTIBILITY IN GUJARAT POPULATION

4A.1. Introduction

Vitiligo is a common depigmenting disorder resulting from the loss of melanocytes in the skin and affects 1-4% of the world population (Ortonne and Bose 1993). The incidence of vitiligo is found to be 0.5-2.5% in India (Das et al 1985; Handa and Kaur 1999). Gujarat and Rajasthan states have the highest prevalence i.e., ~8.8% (Valia and Dutta 1996). Though vitiligo is extensively addressed in the past five decades, its etiology is still being debated (Taieb 2000; Le Poole 1993; Ortonne and Bose 1993; Shajil et al 2006a). Several hypotheses were proposed about the pathogenesis of vitiligo and the major ones are oxidative stress, autoimmune and neurochemical hypotheses. Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction (Schallreuter et al 1999; Schallreuter et al 1994) 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 reported. 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. We reported systemic oxidative stress in vitiligo patients due to an imbalance in the enzymatic and non-enzymatic antioxidant systems (Agrawal et al 2004; Shajil and Begum 2006).

Several studies suggest that genetic factors also contribute to vitiligo susceptibility (Bhatia et al 1992; Nordlund 1997; Kim 1998). About 20% of vitiligo patients have at least one first-degree relative affected (Bhatia et al 1992; Nath et al 1994), indicating that genetic factors are involved in the vitiligo susceptibility. Our studies also suggest that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil et al 2006b). The relative risk of vitiligo for the first-degree relatives of

patients is increased by 7-10 fold (Nath et al 1994). The inheritance pattern of vitiligo does not follow the simple Mendelian pattern. Its mode of heredity suggests that it is a polygenic disease, governed by a set of recessive alleles situated at several unlinked autosomal loci, which may be involved in the antioxidant defense mechanism, melanin synthesis, autoimmunity etc. that could collectively confer the vitiligo phenotype (Nath et al 1994). However, the genetic basis of vitiligo pathogenesis is still an enigma. Several candidate genes are reported for vitiligo such as *CAT, CTLA4, MHC, SLE, ACE, COMT, GTP* cyclohydrolase etc (Fain et al 2003). We attempted a case-control study for the well documented *CAT* exon 9 T/C polymorphism (rs769217) (Casp et al 2002) and its relation to catalase activity and lipid peroxidation levels in Gujarat vitiligo patients where the prevalence of vitiligo is very high. The catalase gene is of 34 kb in length and consists of 12 introns, 13 exons and encodes for a protein of 526 amino acids (Goth et al 2004). The *CAT* gene was selected as a candidate gene because reduction in catalase activity is reported in the epidermis (Schallreuter et al 1999; Schallreuter et al 1991), blood of segmental vitiligo (Shajil and Begum 2006) and peripheral blood mononuclear cells (Dell'Anna 2003) of vitiligo patients. Many allelic variants of *CAT* have also been reported (Goth et al 2004).

4A.2. Materials and methods

For DNA isolation and estimation of antioxidant parameters 5 ml blood was collected from 140 vitiligo patients after written informed consent was obtained. The patients had no other associated diseases. The study included 143 age- matched healthy consenting volunteers as controls.

Genomic DNA was prepared from venous blood of vitiligo patients and controls by using DNA isolation kit (Bangalore Genei, India). The amplified region consisted of the sequence of exon 9 (139 bps) and 63 bps of intron 9 of catalase gene as shown below:

5′GCCGCCTTTTTGCCTATCCTGACACTCACCGCCATCGCCTGGGACCCAATTATC
TTCATATACCTGTGAACTGTCCCTACCGTGCTCGAGTGGCCAACTACCAGCGTGA
[C/T]GGCCCGATGTGCATGCAGGACAATCAGGGTAGGCCTAAAGACGTTGGGCT
CCCCCTGCGTGGGCAGAGGGCACGTGGAGCAGATGGGCGGGA3′.

Oligonucleotide primers (Sigma, USA) used for PCR amplification were 5′ GCCGCCTTTTTGCCTATCCT 3′ (Forward primer) and 5′ TCCCGCCCATCTGCTCCAC 3′ (Reverse primer). PCR amplification was performed in 25 µl reaction system containing 50 ng of genomic DNA and 2 µmole/l of each primer.

For restriction fragment length polymorphism analysis, PCR amplified products were digested with the *Bst* XI (MBI Fermentas, Germany). The recognition sequence of *Bst* XI is given below:

5′...C C A N N N N N↓ N T G G...3′
3′...G G T N↑ N N N N N A C C...5′

The digested products were separated on 15% polyacrylamide gel along with 100 bp DNA ladder. Ethidium bromide stained gels were visualized under UV light.

Erythrocyte catalase activity in the hemolysate was assayed by the standard method (Aebi 1984). Catalase activity is expressed in terms of *k*/g hemoglobin/s, where *k* is the velocity constant of the decomposition H_2O_2 to water.

Erythrocyte lipid peroxidation was estimated according to the standard procedure (Beuge and Aust 1978) and the results are expressed as nmoles of MDA formed/gram hemoglobin/minute.

Statistical analysis

Results of catalase activity and LPO levels in vitiligo patients and age-matched controls were compared using the paired students' t test utilizing statistical program Prism 3 and $p \leq 0.05$ was considered significant. For case/control association studies the significance of observed differences in allelic

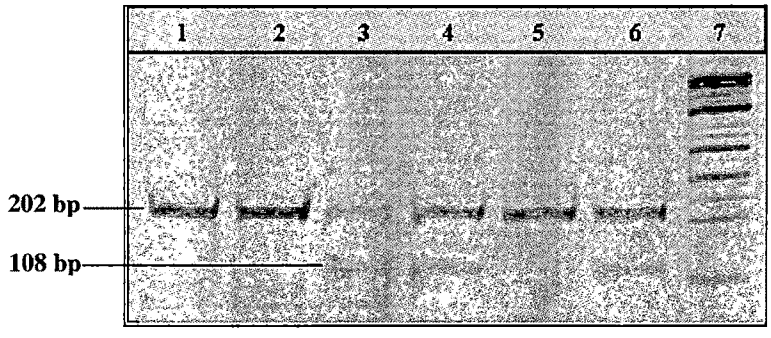
or genotypic frequencies between vitiligo patients and control population was determined by the standard X^2 test.

4A.3. Results

Oxidative stress is known to play a major role in the pathogenesis of vitiligo and catalase among other antioxidant enzymes could contribute in precipitating the disease in susceptible population.

The aim of this study was to find out whether *CAT* exon 9 T/C polymorphism is associated with Gujarat vitiligo patients and to correlate this polymorphism with catalase activity and the corresponding changes in the lipid peroxidation levels in vitiligo patients compared to controls. The polymorphism studied was T/C silent substitution in *CAT* exon 9 (Asp 389) and it was genotyped by RFLP analysis of PCR amplified genomic fragment (202 bp) using the restriction endonuclease *Bst* XI (CCANNNNNNTGG) and the results are shown in Figure 1. The T allele is cleaved by *Bst* XI whereas C allele remains uncut (Figure 1).

Figure 1. Restriction analysis of T→ C (Asp → Asp) polymorphism in the exon 9 of *CAT* gene.



Lanes 1-6: samples after digestion with *Bst XI*. Lanes 1, 2, 5 show C/C genotype; Lanes 3, 4, 6 show C/T genotype. Lane 7 shows molecular weight marker (100 bp DNA ladder).

The observed allele frequencies of C and T were 0.867 and 0.133 respectively in controls; 0.825 and 0.175 in vitiligo patients (Table 1). The allele frequencies of this T/C SNP did not differ significantly between the control and patient population ($p<0.328$). When the observed control and patient genotype

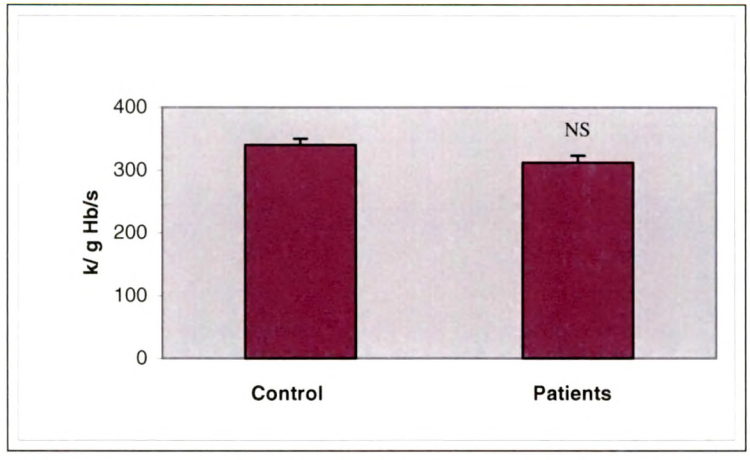
frequencies were compared with the expected values using a 3x2 contingency table in a standard X^2 test (Table 1) they did not show any significant change (p= 0.2988) suggesting that there is no possible association of the T/C exon 9 (*Bst* XI) *CAT* marker with vitiligo (Shajil et al 2007). The observed genotype frequencies of the control population did not differ significantly from those predicted by Hardy Weinberg equation (p<0.259). Also the patient population did not deviate from Hardy Weinberg equilibrium (p<0.0614) (Table 1).

Table 1. Distribution of genotypes and alleles for the T/C SNP in *CAT* exon 9 in vitiligo patients and control population

		Observed genotype counts			Observed allele frequencies		Expected genotype counts			
	N	C/C	C/T	T/T	C	T	C/C	C/T	T/T	p value
Controls	143	106	36	1	0.867	0.133	107.54	32.89	2.57	0.259
Patients	140	92	47	1	0.825	0.175	95.34	40.46	4.08	0.0614
p value								0.2988		

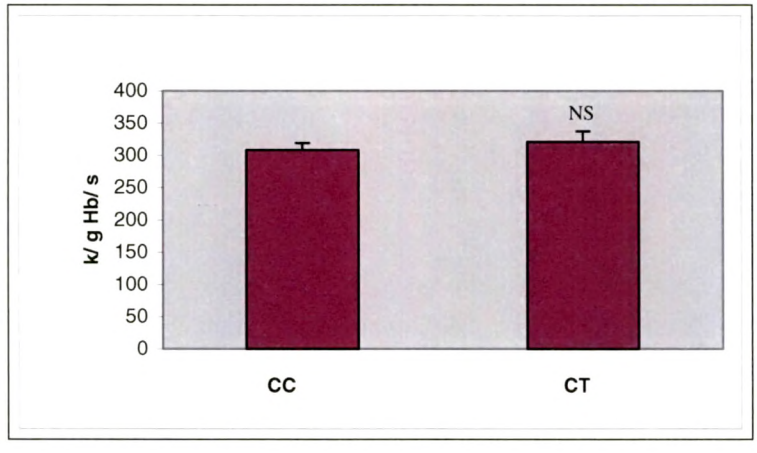
We have also analyzed the catalase activity and lipid peroxidation levels in order to study their correlation in vitiligo patients with respect T/C SNP. Catalase activity did not show any change in vitiligo patients compared to controls (Figure 2). Also, catalase activity did not differ significantly between patients with C/C and C/T genotypes (Figure 3). Lipid peroxidation levels showed significant increase (p<0.0001) in vitiligo patients compared to controls, which is an index for oxidative stress (Figure 4). However LPO levels did not differ significantly between patients with C/C and C/T genotypes.

Figure 2. Erythrocyte catalase activity in controls and vitiligo patients#



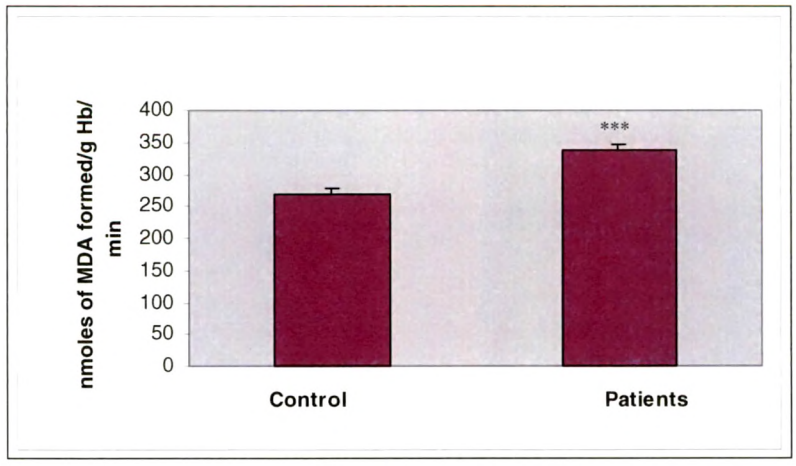
Values are given as mean ± SE of 143 and 140 individual observations in controls and vitiligo patients. NS, non significant

Figure 3. Erythrocyte catalase activity in C/C and C/T genotypes of vitiligo patients#



Values are given as mean ± SE of 92 and 47 individual observations in C/C and C/T genotypes of vitiligo patients. NS, non significant.

Figure 4. Erythrocyte LPO levels in controls and vitiligo patients.



Values are given as mean ± SE of 143 and 140 individual observations in controls and vitiligo patients. ***, $p<0.001$

4A.4. Discussion

Vitiligo susceptibility is a complex genetic trait that may involve genes involved in melanin biosynthesis, response to oxidative stress and regulation for autoimmunity. Environmental factors may also contribute for vitiligo susceptibility.

We have selected *CAT* as a candidate gene to study vitiligo susceptibility and our results suggest that there is no association of T/C *CAT* exon 9 SNP in Gujarat vitiligo patients compared to controls. However Casp et al (2002) showed an association between vitiligo susceptibility and T/C SNP at codon 389 in Caucasian population. Gavalas et al (2006) also reported an association between this SNP with vitiligo susceptibility in English population. However, in Korean population the T/C *CAT* exon 9 genotype distribution and allele frequencies were not significantly associated with vitiligo (Park et al 2006), which is in accordance with our study. Thus it seems that the genotype distribution of the *Bst XI* is different in different ethnic groups.

Catalase is a heme containing enzyme that catalyses the conversion of H_2O_2 to water and oxygen, thereby preventing cell damage from highly reactive oxygen derived free radicals (Goth et al 2004). This study shows that there is no

change in the catalase activity in vitiligo patients compared to controls and our results are in accordance with our previous studies (Agrawal et al 2004; Amina et al 2006) as well as other reports (Hazneci et al 2005; Dell'Anna et al 2001). However, significant reduction in catalase activity is shown in segmental vitiligo patients compared to controls (Shajil and Begum 2006). There are also other reports showing significant reduction in catalase activity in vitiligo patients compared to controls (Schallreuter et al 1991; Dell'Anna et al 2001; Passi et al 1998). In patients C/C and C/T genotypes have not shown any significant change in the catalase activity.

We have observed a significant increase in lipid peroxidation levels in vitiligo patients compared to controls. There are several studies that showed significant increase in LPO levels in vitiligo patients compared to controls (Ines et al 2006; Koca et al 2004; Agrawal et al 2004; Yildirim et al 2003; Shajil et al 2006c; Picardo et al 1994). In patients C/C genotype showed higher LPO levels ($p<0.012$) compared to C/T genotype.

Oxidative stress plays a major role in the pathogenesis of vitiligo. Oxidative stress in vitiligo patients may be generated due to several perturbed biochemical pathways and thus leads to accumulation of H_2O_2. Low catalase levels have also been reported in the epidermis of vitiligo patients. In addition, allelic variants are reported in the *CAT* gene with low levels of catalase activity (Goth et al 2004). However, it is not yet established how the *CAT* gene T/C SNP could result in low catalase levels (Gavalas et al 2006). Interestingly, our study does not show any significant change in the catalase activity of CC and C/T genotypes in the patients and controls.

The present study shows that the well-documented *CAT* exon 9 T/C polymorphism in Caucasian population may not be associated with Gujarat vitiligo patients where the prevalence of vitiligo is alarmingly high. These results suggest for the presence of novel SNPs in Gujarat vitiligo population.

4A.5. References

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CHAPTER IV B

ASSOCIATION OF *GLUTATHIONE PEROXIDASE* CODON 200 POLYMORPHISM IN RELATION TO ITS ACTIVITY AND OXIDATIVE STRESS WITH VITILIGO SUSCEPTIBILITY IN GUJARAT POPULATION

4B.1. Introduction

Vitiligo, a cosmetic disfigurement disorder is a global problem and its clinical hallmark is loss of melanin pigment due to decreased number of functioning melanocytes or their complete absence in the lesional skin. Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction (Schallreuter et al 1999; Schallreuter et al 1994) 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 glutathione peroxidase 1 activity has been demonstrated in vitiligo patients compared to controls (Beazley et al 1999; Yildirim et al 2003; Agrawal et al 2004; Shajil and Begum 2006; Ines et al 2006). The importance of genetic factors for vitiligo susceptibility is evident by reports of significant familial association from several laboratories (Bhatia et al 1992; Nordlund 1997; Kim 1998). Several candidate genes are reported for vitiligo such as catalase, cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), major histocompatibility complex (*MHC*), angiotensin converting enzyme (*ACE*), catechol O methyltransferase (*COMT*), *GTP* cyclohydrolase etc (Fain et al 2003). However, the role of genetic factors in vitiligo pathogenesis is still not well understood.

Glutathione peroxidase 1 is a selenium dependent enzyme that participates in the detoxification of H_2O_2 and lipoperoxides and is ubiquitously expressed (Arthur 2000). The *GPX* 1 gene is located on chromosome 3p21.3. The glutathione peroxidase 1 gene is 1.424 kb in length and contains 1 intron, 2 exons and encodes for a protein of 201 amino acids (Ishida et al 1987). The proline to leucine at codon 200 polymorphism (rs1050450) in *GPX* 1 gene is reported to result in decreased glutathione peroxidase activity (Hu and Diamond 2003). Our results on antioxidant status showed a significant decrease in glutathione peroxidase activity in vitiligo patients compared to controls (Agrawal et al 2004), hence we selected *GPX* 1 gene to study its genetic association with vitiligo. We attempted a case-control study to evaluate the association of the proline to leucine polymorphism at codon 200 of *GPX 1* with vitiligo. The *GPX* 1 activity

and lipid peroxidation levels were also estimated to correlate the oxidative stress hypothesis with respect to this polymorphism in Gujarat vitiligo patients.

4B.2. Materials and methods

For DNA isolation, estimation of glutathione peroxidase activity and lipid peroxidation levels, 5 ml blood was collected from 126 vitiligo patients after written informed consent was obtained. The patients had no other associated diseases. The study included 149 age matched healthy consenting volunteers as controls. Genomic DNA was prepared from venous blood of vitiligo patients and controls by using DNA isolation kit (Bangalore Genei, India). The amplified region consisted of the sequence of exon 2 (303 bps) and 34 bps of 3′ flanking region of glutathione peroxidase gene as shown below.

5′TGTGCCCCTACGCAGGTACAGCCGCCGCTTCCAGACCATTGACATCGAGCCTG
ACATCGAAGCCCTGCTGTCTCAAGGGC[C/T]CAGCTGTGCCTAGGGCGCCCCTCC
TACCCCGGCTGCTTGGCAGTTGCAGTGCTGCTGTCTCGGGGGGGTTTTCATCTAT
GAGGGTGTTTCCTCTAAACCTACGAGGGAGGAACACCTGATCTTACAGAAAATA
CCACCTCGAGATGGGTGCTGGTCCTGTTGATCCCAGTCTCTGCCAGACCAAGGC
GAGTTTCCCCACTAATAAAGTGCCGGGTGTCAGCAGAACTGTGTGTATGTCCTGT
GTCATTGTCATTTGG 3′

Oligonucleotide primers (MWG, Bangalore) used for PCR amplification were 5′ TGTGCCCCTACGCAGGTACA 3′ (Forward primer) and 5′ CCAAATGACAATGACACAGG 3′ (Reverse primer). PCR amplification was performed in 25 µl reaction system containing 50 ng of genomic DNA and 2µmol/l of each primer. For restriction fragment length polymorphism analysis, PCR amplified products were digested with the *Apa* I (MBI Fermentas, Germany). The recognition sequence of *Apa* I is given below.

5′…G G G C C ↓C…3′
3′…C↑ C C G G G…5′

The digested products were separated on 2% agarose gel along with 100 bp DNA ladder. Ethidium bromide stained gels were visualized under UV light.

Erythrocyte GPX activity was assayed according to the standard method (Paglia and Valentine 1967). GPX activity is expressed in terms of µmol GSH utilized/g hemoglobin/s. Erythrocyte lipid peroxidation was estimated according to the standard procedure (Beuge and Aust 1978) and the results are expressed as nmoles of MDA formed/gram hemoglobin/minute.

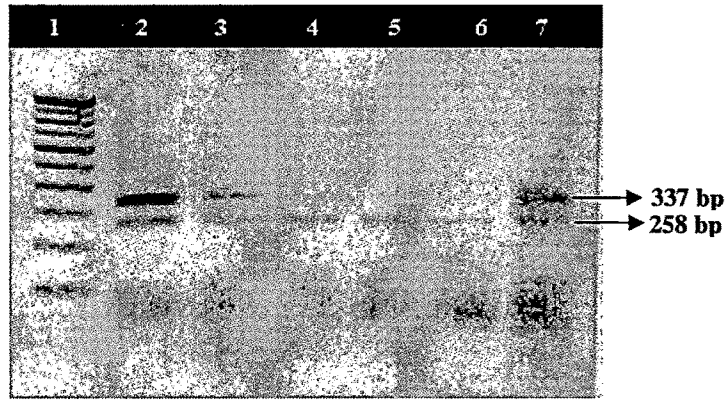
Statistical analysis

Results of glutathione peroxidase and LPO levels in vitiligo patients and age-matched controls were compared using the paired student's t test utilizing statistical software Prism 3 and $p \leq 0.05$ was considered significant. For case/control association studies the significance of observed differences in allelic or genotypic frequencies between vitiligo patients and control population was determined by standard X^2 test.

4B.3. Results

The aim of this study was to analyze the frequency of codon 200 proline to leucine polymorphism within *GPX* 1 gene in Gujarat vitiligo patients and to correlate this polymorphism with glutathione peroxidase activity and the corresponding changes in the lipid peroxidation levels in vitiligo patients compared to controls. The polymorphism studied was C/T substitution in *GPX* 1 exon and was genotyped by RFLP analysis of amplified genomic fragments using the restriction endonuclease *Apa* I and the results are shown in Figure 1. The C allele of *GPX* 1 is cleaved by *Apa* I whereas T allele remains uncut (Figure 1).

Figure 1. Restriction analysis of C→ T (Pro → Leu) polymorphism in the exon 2 of *GPX* gene.



Lanes 1-6: Samples after digestion with *Apa I*. Lanes 2, 3, 7 show C/T genotype; Lanes 4, 5, 6 show C/C genotype. Lane 1 shows molecular weight marker (100 bp DNA ladder)

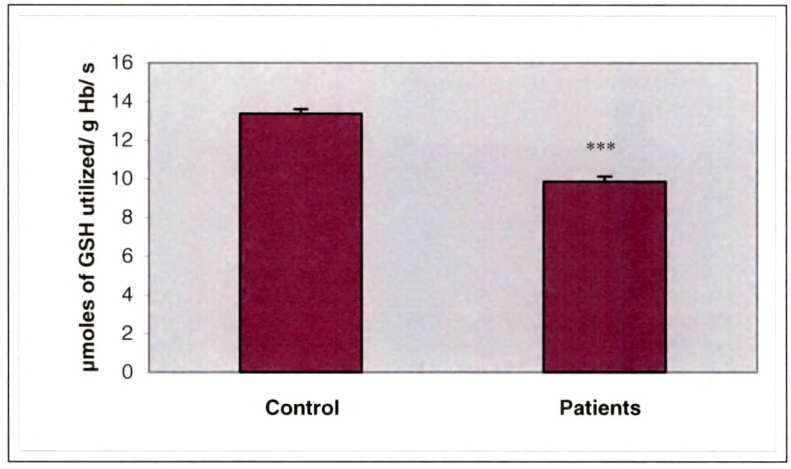
The observed *GPX* 1 allele frequencies of C and T were 0.711 and 0.289 respectively in controls; 0.742 and 0.258 in vitiligo patients (Table 1). The allele frequencies of this C/T SNP did not differ significantly between the control and patient population ($p<0.568$). When the observed control and patient genotype frequencies were compared with the expected values using a 3x2 contingency table in a standard X^2 test (Table 1) they did not show any significant change ($p= 0.3430$) suggesting that there is no association of the C/T exon 2 (*Apa* 1) *GPX* 1 marker with vitiligo (Shajil et al 2007). The observed genotype frequencies of the control population differed significantly from those predicted by Hardy Weinberg equation ($p<0.0304$). Also the patient population is deviated from Hardy Weinberg equilibrium ($p<0.0037$) (Table 1).

Table 1. Distribution of genotypes and alleles for the C/T SNP in *GPX 1* exon 2 in vitiligo patients and control populations

		Observed genotype counts			Observed allele frequencies		Expected genotype counts			
	N	C/C	C/T	T/T	C	T	C/C	C/T	T/T	p value
Controls	149	70	72	7	0.711	0.289	75.24	61.24	12.52	0.0304
Patients	126	63	61	2	0.742	0.258	69.3	48.26	8.44	0.0037
p value								0.3430		

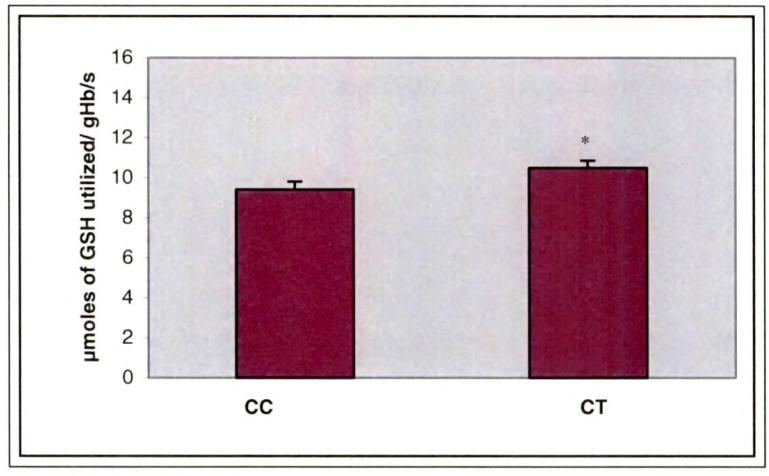
We have also analyzed the glutathione peroxidase activity and lipid peroxidation levels in vitiligo patients and controls in order to study their correlation in vitiligo patients with respect to C/T SNP. Glutathione peroxidase was found to be significantly decreased ($p<0.0001$) in vitiligo patients compared to controls (Figure 2). Also, glutathione peroxidase activity was significantly decreased ($p<0.0486$) in patients with C/C genotype compared to C/T genotype (Figure 3). Lipid peroxidation levels showed significant increase ($p<0.0001$) in vitiligo patients compared to controls (Figure 4). However LPO levels did not differ significantly between patients with *GPX* 1 C/C and C/T genotypes.

Figure 2. Erythrocyte glutathione peroxidase activity in controls and vitiligo patients#



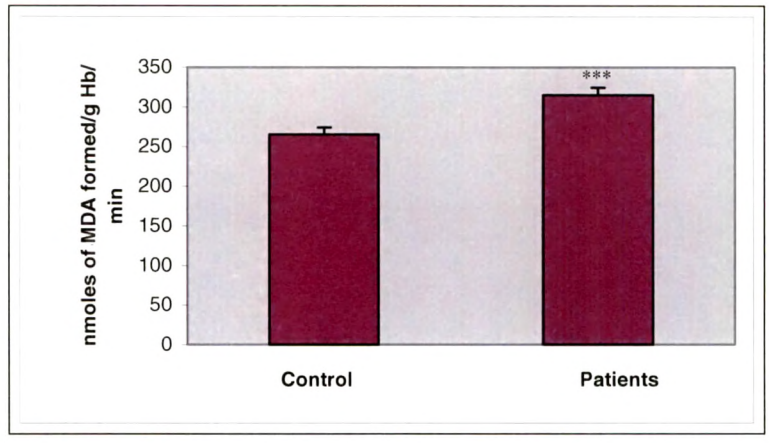
Values are given as mean ± SE of 149 and 126 individual observations in controls and vitiligo patients. ***, p<0.001

Figure 3. Erythrocyte glutathione peroxidase activity in C/C and C/T genotypes of vitiligo patients#



Values are given as mean ± SE of 63 and 61 individual observations in C/C and C/T genotypes of vitiligo patients. *, p<0.05

Figure 4. Erythrocyte LPO levels in controls and vitiligo patients#



Values are given as mean ± SE of 149 and 126 individual observations in controls and vitiligo patients. ***, $p<0.001$

4B.4. Discussion

Vitiligo susceptibility is a complex genetic trait, which is characterized by incomplete penetrance, multiple susceptibility loci and genetic heterogeneity (Zhang et al 2005). The single nucleotide polymorphisms present in the genes involved in melanin biosynthesis, response to oxidative stress and regulation for autoimmunity may be playing a major role in the pathogenesis of vitiligo in the susceptible population.

We have selected *GPX* 1 as a candidate gene to study vitiligo susceptibility and our results suggest that there is no association of C/T *GPX 1* exon 2 SNP in Gujarat vitiligo patients compared to controls. Nevertheless, Ratnasinghe et al (2000) showed that the codon 200 *GPX* 1 polymorphism is associated with increased risk of lung cancer in Finnish population. It was also reported that leucine containing *GPX* 1 allele was more frequently associated with breast cancer than the proline containing allele (Hu and Diamond 2003). It was also reported that proline containing *GPX* 1 allele exhibited low glutathione peroxidase activity compared to leucine containing allele (Hu et al 2003). Conversely, our study could not find any association of this polymorphism with vitiligo, however the proline containing *GPX*1 allele (C/C) showed significant reduction ($p<0.0486$) in glutathione peroxidase activity in vitiligo

patients compared to patients with C/T genotype. Interestingly, this change in activity was not observed in C/C and C/T genotypes of control population.

This study also shows a significant increase in lipid peroxidation levels in vitiligo patients compared to controls. There are several reports that showed significant increase in LPO levels in vitiligo patients compared to controls (Ines et al 2006; Koca et al 2004; Agrawal et al 2004; Yildirim et al 2003; Shajil et al 2006; Picardo et al 1994). However, no change is observed in lipid peroxidation levels in vitiligo patients with different genotypes.

Oxidative stress plays a major role in the pathogenesis of vitiligo. Oxidative stress in vitiligo patients may be generated due to several perturbed biochemical pathways and thus leads to accumulation of H_2O_2. Low glutathione peroxidase levels have also been reported in vitiligo patients compared to controls. In addition, allelic variants are reported in the *GPX 1* gene with low levels of glutathione peroxidase activity (Hu and Diamond 2003; Ratnasinghe et al 2000). The low activity of glutathione peroxidase of proline allele (C/C) may be due to the fact that different *GPX 1* alleles encode structurally different GPX 1 protein subunits. Hence, heterozygote individuals may be having a less efficient final glutathione peroxidase complex. The polymorphism of substitution of the α-imino acid proline by leucine is probably causing the most profound secondary and tertiary conformational changes in GPX 1 protein, because proline is the only amino acid without a free unsubstituted amino group on the α carbon atom and is known to cause a unique kink in the secondary structure of the peptides (Ratnasinghe et al 2000). Our study also shows a significant decrease in the glutathione peroxidase activity in C/C genotype compared to C/T genotype in vitiligo patients.

The present study shows that the well documented *GPX 1* exon 2 C/T polymorphism association with cancer may not be associated with Gujarat vitiligo patients where activity of GPX 1 is significantly decreased. These results suggest for the presence of novel SNPs in Gujarat vitiligo population.

4B.5. References

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