4.1 INTRODUCTION

Vitiligo is an acquired depigmentation disorder characterized by the milky white patches on the skin due to the loss of functional melanocytes. Its incidence rate is approximately 0.1–2.0% in the world and 0.1 to 8.8% in India (Taieb *et al*., 2007; Sehgal and Srivastava, 2007). The precise *modus operandi* for pathogenesis of vitiligo has remained obscure. The onset and progression of vitiligo are thought to be driven by multiple inherited genes and environmental triggers (Spritz, 2006; Laddha *et al*., 2013a). Various studies including ours have suggested that oxidative stress caused due to hydrogen peroxide (H_2O_2) accumulation (Schallreuter *et al*., 1999; Spencer *et al*., 2007), low catalase (CAT), glutathione peroxidase (GPx) (Agrawal *et al*., 2004; Shajil and Begum, 2006; Shajil *et al*., 2007; Mansuri *et al*., 2016), glucose-6-phosphate dehydrogenase (G6PD) levels (Arican and Kurutas, 2008; Farahi-Jahromy *et al*., 2014) and high superoxide dismutase (SOD) (Laddha *et al*., 2013b) and lipid peroxidation (LPO) (Laddha *et al*., 2014) levels in the skin and/ blood, is a crucial factor in melanocyte destruction in vitiligo. Genetic variations have been implicated in susceptibility to vitiligo by numerous researchers (Al-Shobaili, 2011; Spritz, 2012, 2013). Our previous studies on genetic variations in antioxidant enzyme genes showed association of polymorphisms in *GPX1*, *SOD2* and *SOD3* with vitiligo susceptibility in Gujarat population (Laddha *et al*., 2013b; Mansuri *et al*., 2016).

G6PD plays an important role in protecting cells from oxidative damage by producing reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced glutathione (Alharbi and Khan, 2014). The NADPH in turn maintains the supply of reduced glutathione (GSH) and catalase in the cells that are used to detoxify free radicals and H_2O_2 (Scott *et al*., 1993). Various studies including ours have reported decreased G6PD levels in vitiligo patients (Agrawal *et al*., 2004; Shajil and Begum, 2006; Arican and Kurutas, 2008; Farahi-Jahromy *et al*., 2014). However, there is no information about the molecular basis of G6PD deficiency in vitiligo. Variations in *G6PD* gene lead to G6PD deficiency; most of which are single amino acid substitutions and the resulting G6PD variants have differential enzyme activity and were found to be associated with a variety of clinical phenotypes, resulting from an increased susceptibility of individuals to oxidative stress (Tishkoff *et al*., 2001; Cappellini and Fiorelli, 2008). Amini and Ismail

(2003) have reported the association of a SNP in 3'-UTR of *G6PD* (rs1050757) and its possible role in mRNA secondary structure with G6PD deficiency. miRNAs regulate gene expression at the posttranscriptional level by binding to the 3′- UTR of target mRNAs (Filipowicz *et al*., 2008). Our previous study on skin miRNA profiling showed increased expression of miR-1 in lesional skin of vitiligo patients (Mansuri *et al*., 2014a; 2014b), which is known to target *G6PD* (Xu *et al*., 2007; Singh *et al*. 2013). However, so far there is no report on *G6PD* with respect to polymorphisms and/ or its regulation via miRNA in vitiligo.

Therefore, the present study was aimed 1) to investigate *G6PD* exon 2 G/C (Gln41His; rs1050827), exon 4 G/T (Val107Leu; rs11555344), exon 5 G/A (Asp143Asn; rs5030870), exon 5 I/D (C/-; Met155; rs34233392), exon 6 C/T (Ser218Phe; rs5030868); exon 9 G/C (Asp380His; rs34193178), exon 10 G/A (Gln402Gln; rs2230036), exon 11 C/T (Tyr467Tyr; rs2230037) and 3' UTR A/G (rs1050757) polymorphisms; 2) to estimate G6PD activity and LPO levels in erythrocytes; 3) to estimate *G6PD* and hsa-miR-1 expression levels in skin and blood of Gujarat vitiligo patients and controls.

4.2 MATERIALS AND METHODS

4.2.1 Study subjects

Both vitiligo patients and controls were from Gujarat state of India (Table 1). Vitiligo patients did not receive 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 snapfrozen, from lesional and non-lesional skin of patients with vitiligo (n= 12) and from non-inflamed, non-irritated skin of individuals (n= 12). Further, we recruited 366 vitiligo patients and 449 controls for blood sample collection. Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, USA). The importance of our study was explained to all the 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 = 366)	(n = 449)
Average age (mean age ± SD)	29.38± 4.480 yr	28.33± 3.240 yr	30.92 ± 12.15 yr	29.11 ± 11.96 yr
Sex: Male	4	6	106 (28.96%)	144 (32.07%)
Female	8	6	260 (71.04%)	305 (67.93%)
Age of onset (mean age ± SD)	27.83 ± 6.05 yr	NA	21.76 ± 11.93 yr	NA
Duration of disease (mean ± SD)	6.83 ± 2.36 yr	NA	8.21 ± 7.24 yr	NA
Type of vitiligo				
Active vitiligo	7	NA	271 (74.04%)	NA
Stable vitiligo	5	NA	95 (25.96%)	NA
Localized vitiligo	4	NA	327 (89.34%)	NA
Generalized vitiligo	8	NA	39 (10.66%)	NA

4.2.2 Estimation of G6PD enzyme activity:

Erythrocyte G6PD activity was assayed by the method of Kornberg and Horecker (1955) by measuring increase in the absorbance at 340 nm, which occurs due to the reduction of NADP to NADPH when two electrons were transferred from glucose 6 phosphate to NADP in the reaction catalyzed by glucose 6 phosphate dehydrogenase. Briefly, take 100 µl of Tris HCl Buffer (50 mM, pH 7.6), 150µl of 10% Triton X100, 100 µl of $MgCl_2$ (0.01 M), 300 µl of NADP (2mM), 600 µl of Glucose 6 phosphate (6mM), 100 µl of sample hemolysate (1g Hb/dl) and make up to 2ml using double distilled water into a vial. Immediately after adding $G6PO_4$ the absorbance was recorded at every 5 seconds of interval for 3 minutes at 340nm. Blank was run for each sample which contained all above reagents except hemolysate. All chemicals used in assay were procured from

SIGMA Chemical Co, St. Louis, Missouri, USA. G6PD activity was expressed as mmoles of NADP reduced to NADPH/min/g Hb. LPO levels were estimated according to the Beuge and Aust method (1978), as described earlier in 'Chapter II'.

4.2.3 Genomic DNA Isolation and Genotyping of *G6PD* 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 for genotyping of rs1050827, rs11555344, rs34233392, rs5030868, rs2230036, rs2230037, rs34193178 and rs1050757 SNPs whereas, amplification refractory mutation system (ARMS)-PCR method was used for genotyping of rs5030870 SNP. 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'. Particularly, in case of rs1050757, two step PCR was performed to avoid the non-specific amplification: first 398 bp product was amplified using outer primer set and genomic DNA as template; in second step the PCR product of 398 bp was used as template and 126 bp amplicon was amplified using inner set of primers, which was further subjected for digestion with restriction enzyme (Table 2).

4.2.4 RNA isolation and cDNA synthesis

Total RNA from skin biopsies and whole blood was extracted using mirVana® miRNA isolation kit (Ambion®, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA synthesis for miRNA expression study was done using TaqManH MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems®, Foster City, California, USA). cDNA synthesis for mRNA expression study was performed using the Verso cDNA Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions in the Mastercycler Gradient PCR (Eppendorf, Germany).

4.2.4 miRNA/mRNA expression

TaqMan® Low Density Assay (Applied Biosystems®, Foster City, California, USA) was used to estimate the expression of miR-1 in whole blood of vitiligo patients and controls. RT product was introduced into PCR reactions which were incubated on the ABI

Table 2. Primers and restriction enzymes used for genotyping of *G6PD* polymorphisms and expression.

Gene/ SNP	Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme	Digested Products (bp)
rs1050827	FP	CGGAATACACCAATGCTTTGAGT	65	499	*Tai*I	297+202
	RP	GAGGAGGAGCTCAACTTAGCA				
rs11555344	FP	CATCTGGTAAGTGTGTCCCACC	71	448	***Taa*I**	277+171
	RP	TGACACCCAACTATGATTGGCG				
rs5030870	FP1	TCCTATGTGGCTGGCCAGTAC**A**	63	493	--	--
	FP2	TCCTATGTGGCTGGCCAGTAC**G**				
	RP	ACCTTTGCTTTACTACCCCCGC				
hGH (PCR internal control)	FP	CTA TGC TCC GCG CCC ATC GT	69	407	--	--
	RP	TGG GGA GAA GGC ATC CAC TCA CG				
rs34233392	FP	TGG TGG GAG CAC TGC CTG GGC	71	198	BccI	181+17
	RP	CTA CCA GCG CCT CAA CAG CCA **G**AT				
rs5030868	FP	TCA AGG GGG TAA CGC AGC TC	64	396	MboII	295+101
	RP	TGG GGC CGA AGA TCC TGT TG				
rs2230036	FP	AGCCGTCGTCCTCTATGTGG	61	353	PstI	241+112
	RP	CGGCTTCTTGGTCATCATCTTG				
rs2230037	FP	TGTTCTTCAACCCCGAGGAGT	65	207	***Bcl*I**	183+24
	RP	AAGACGTCCAGGATGAGG**T**G**A**TC				
rs34193178	FP	GCAGAGCCAAGCAGGGGCCT	67	363	NlaIII	304+43+16
	RP	GTGCCCGCACACAGGGCATG				
rs1050757	'O'FP	TCGCTGCTGCTACTACCCG	60	398	--	
	'O'RP	AGAGAGGCTGCCTACGGGAG				
	'I'FP	CC AGG GTG GGA GGG AGG **CT**C	67	126	***Xho*I**	98+18
	'I'RP	TCG AGA TGT TGC TGG TGA CAA GG				
G6PD expression	FP	TGAGCCAGATAGGCTGGAA	63	225	--	--
	RP	TAACGCAGGCGATGTTGTC				
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; 'O': outer primer; 'I': inner primer.

QuantStudio thermocycler (Applied Biosystems®, Foster City, California, USA) at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. miRNA expression data was normalized with U6 snRNA. The transcripts levels of *G6PD* 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. Details are described in 'Chapter II'.

4.2.5 Bioinformatics analysis:

We employed the *in silico* prediction tools like: SIFT (Kumar *et al*., 2012), PANTHER (Thomas *et al*., 2003), I-MUTATNT (Capriotti *et al*., 2008), POLYPHEN (Adzhubei *et al*., 2010), MUPRO (Cheng *et al*., 2006) to predict the impact on the protein due to single amino acid variations. SNPs and GO (Capriotti *et al*., 2006) predicts the variation effect which might terminate into a disease like a trait. Details are described in 'Chapter II'. miRNA binding sites in 3' UTR region of G6PD gene were predicted using *in silico* target prediction tool: TargetScan (http://www.targetscan.org).

4.2.6 Data analyses

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *G6PD* 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. G6PD activity, LPO levels, relative *G6PD* expression were compared between different groups and genotype-phenotype correlation was analyzed and plotted by nonparametric 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 miRNA/ 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).

4.3 RESULTS

4.3.1 miR-1 expression in vitiligo patients:

Vitiligo patients showed 5.82 fold increased expression of miR-1 in whole blood, as compared to controls (Mean ΔCt ± SEM 15.19±0.383 vs. 17.73±0.6923; p=0.0119) (Figure 1). Further, analysis based on type of vitiligo and disease progression demonstrated that generalized vitiligo (GV), localized vitiligo (LV) and active vitiligo (AV) patients showed significant increase in expression of miR-1 as compared to controls (Mean ΔCt ± SEM: 15.9±0.3997, 15.83± 0.5659 and 15.97± 0.4153 vs. 17.73±0.6923 respectively) (p=0.0258, p=0.0462 & p=0.0268 respectively; Figure 1). However, no significant difference was observed for miR-1 in case of stable vitiligo (SV) vs. controls (Mean ΔCt ± SEM: 17.02±1.013 vs. 17.73±0.6923; p=0.2268). Also, there was no significant difference between LV and GV patients (p=0.9206) as well as between SV and AV patients for miR-1 expression (p=0.3878). Moreover, analysis based on gender showed no significant difference between male and female patients for miR-1 expression (Mean ΔCt ±SEM: 16.78±0.5813 vs. 15.83±0.5324; p=0.2625; Figure 1).

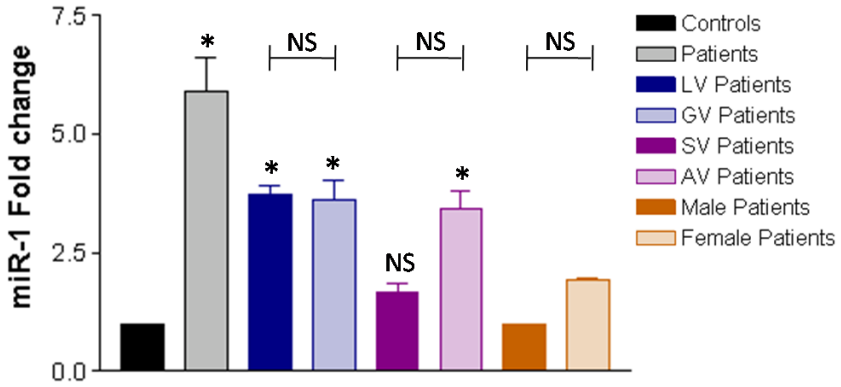

Figure 1: miR-1 expression in vitiligo patients: Vitiligo patients showed significantly increased miR-1 expression in whole blood, as compared to controls (p=0.0119). LV, GV and AV patients showed significantly increased miR-1 expression, as compared to controls (p=0.0462, p=0.0258 and p=0.0268 respectively). No difference in miR-1 expression was observed for control vs. SV (p=0.2268); LV vs. GV (p=0.9206); SV vs. AV (p=0.3878) and male vs. female patients (p=0.2625). [AV: Active Vitiligo; SV: Stable Vitiligo; GV: Generalized vitiligo; LV: Localized vitiligo; *p<0.05; NS: non-significant (p>0.05)].

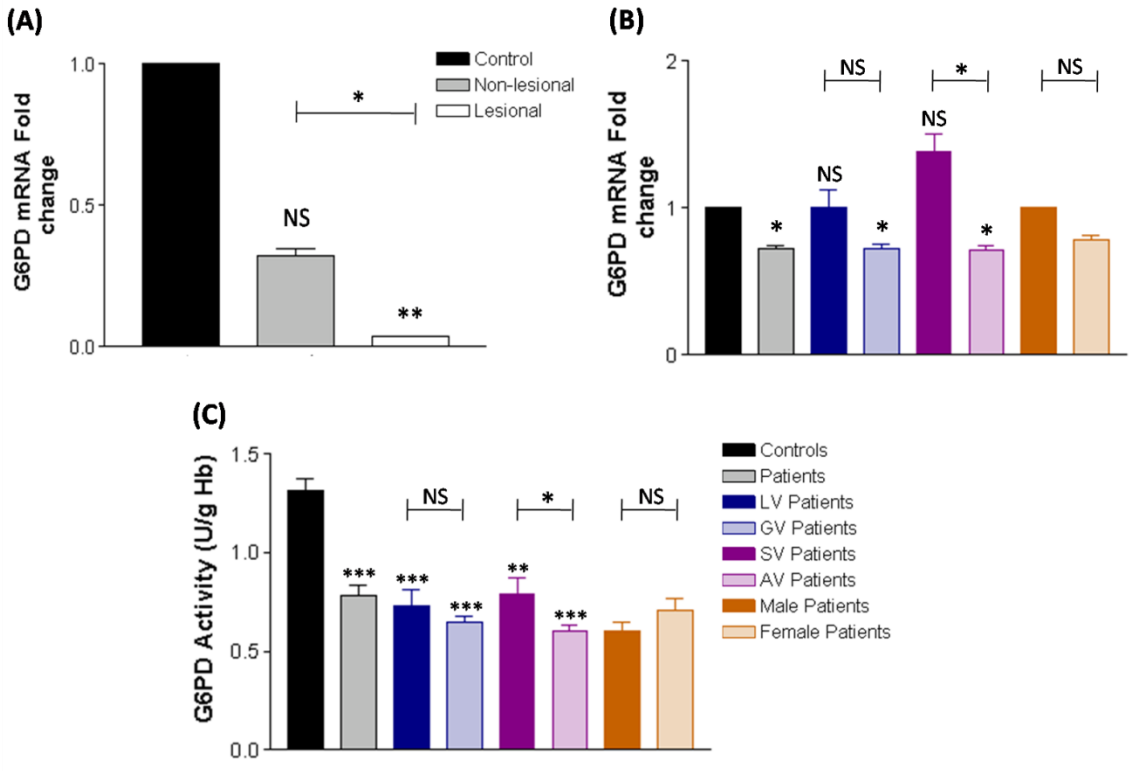

Figure 2: *G6PD* expression and activity in vitiligo patients: (A) Lesional skin of vitiligo patients showed significantly decreased *G6PD* expression as compared to control skin (p=0.0011) as well as non-lesional skin (p=0.0282). No difference in *G6PD* expression was observed for control vs. non-lesional skin (p=0.2511). **(B)** Vitiligo patients showed significantly decreased *G6PD* expression in whole blood, as compared to controls (p=0.0328). GV and AV patients showed significantly decreased *G6PD* expression, as compared to controls (p=0.0327 and 0.0341 respectively). AV patients showed significantly decreased *G6PD* expression, as compared to SV (p=0.0308). No difference in *G6PD* expression was observed for control vs. LV (p=0.9759); control vs. SV (p=0.0841); LV vs. GV (p=0.2646) and male vs. female patients (p=0.3436). **(C)** Vitiligo patients showed significantly decreased G6PD activity in erythrocytes, as compared to controls (p<0.0001). LV, GV, SV and AV patients showed significantly decreased G6PD activity, as compared to controls (p=0.0001, p<0.0001, p=0.0042 and p<0.0001 respectively). AV patients showed significantly decreased G6PD activity, as compared to SV (p=0.016). No difference in G6PD activity was observed for LV vs. GV (p=0.2944) and male vs. female patients (p=0.1198). [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)].

4.3.2 *G6PD* expression in skin of vitiligo patients:

Lesional skin of patients showed significantly decreased mRNA expression of *G6PD* as compared to healthy control skin (Mean ΔCt ±SEM: 17.96±0.8726 vs. 13.14±0.8542; p=0.0011; Figure 2A). Further, lesional skin showed significantly decreased *G6PD* mRNA as compared to non-lesional skin of patients (Mean ΔCt ±SEM: 17.96±0.8726 vs. 14.78±1.038; p=0.0282). However, there was no significant difference in *G6PD* expression between non-lesional skin of patients and healthy control skin (p=0.2511; Figure 2A).

4.3.3 *G6PD* expression in blood of vitiligo patients:

Vitiligo patients showed significantly decreased mRNA expression of *G6PD* in blood as compared to controls (Mean ΔCt ± SEM: 5.415 ± 0.181 vs. 4.954 ± 0.1044; p= 0.0328; Figure 2B), as determined by mean ΔCt. Further, analysis based on type of vitiligo showed that GV patients (Mean ΔCt ± SEM: 5.418 ± 0.1983) exhibit significantly decreased *G6PD* mRNA as compared to controls (p=0.0327). However, there was no significant difference observed in *G6PD* mRNA between LV patients (Mean ΔCt ± SEM: 4.963 ± 0.4045) and controls (p=0.9759), as well as between GV and LV patients (p=0.2646; Figure 2B). Furthermore, analysis based on disease progression demonstrated that AV patients (Mean ΔCt ± SEM: 5.43±0.1979) showed significantly decreased *G6PD* mRNA as compared to SV (Mean ΔCt ± SEM: 4.493±0.317) as well as controls (p=0.0308 and p=0.0341 respectively; Figure 2B). However, there was no significant difference in *G6PD* mRNA between SV patients and controls (p=0.0841). Moreover, analysis based on gender showed no difference in *G6PD* mRNA between male and female patients (Mean ΔCt ± SEM: 4.959±0.2086 vs. 5.314±0.3171; p=0.3436; Figure 2B).

4.3.4 G6PD activity and LPO levels in vitiligo patients:

Vitiligo patients showed significantly decreased G6PD activity as compared to controls (Mean ± SEM: 1.314 ± 0.05986 vs. 0.7835 ± 0.05171; p<0.0001; Figure 2C). Further, analysis based on type of vitiligo showed that GV (Mean ± SEM: 0.6486 ± 0.03341) and LV patients (Mean ± SEM: 0.7325 ± 0.08193) show significantly decreased G6PD

activity as compared to controls (p<0.0001 and p= 0.0001 respectively). However, there was no significant difference in G6PD activity between GV and LV patients (p= 0.2944). Furthermore, analysis based on disease progression demonstrated that AV patients (Mean ± SEM: 0.6012 ± 0.03044) exhibit significantly decreased G6PD activity as compared to SV (Mean ± SEM: 0.7926 ± 0.08183) as well as controls (p=0.016 and p<0.0001respectively; Figure 2C). Also, SV patients showed significant decrease in G6PD activity as compared controls (p=0.0042). Moreover, analysis based on gender showed no difference in G6PD activity between male and female patients (Mean ± SEM: 0.6036 ± 0.04628 vs. 0.7125 ± 0.05259; p= 0.1198; Figure 2C). In addition, vitiligo patients showed significantly increased LPO levels as compared to controls (p<0.0001; data not shown).

4.3.5 Bioinformatics analysis:

The impact of non-synonymous SNPs on the stability and function of the protein was predicted by widely used *in silico* bioinformatics tools (Table 3). PANTHER tool showed G6PD Gln41His (Score= -2.135, $P_{deleterious}$= 0.2963), Val107Leu (Score= -2.95327, $P_{deleterious}$= 0.48832), Asp143Asn (Score= -3.06584, $P_{deleterious}$= 0.51645), Ser218Phe (Score= -4.73175, $P_{deleterious}$= 0.84964) and Asp380His (Score= -4.0413, $P_{deleterious}$= 0.7391) variations to be deleterious for the G6PD function, with the highest score of -4.73175 and probability of ~85% for S218F variation (Table 3). SIFT tool revealed G6PD Gln41His (Score= 0.048), Ser218Phe (Score= 0.008) and Asp380His (Score= 0.022) variations to be deleterious for the G6PD, while Val107Leu (Score= 0.361), Asp143Asn (Score= 0.092), Gln402Gln (Score= 1) and Tyr467Tyr (Score= 1) were predicted to be tolerated (Table 2). POLYPHEN tool showed Gln41His (Score=0.973, Sensitivity=0.77, Specificity=0.96) and Asp380His (Score=0.981, Sensitivity=0.75, Specificity=0.96) substitutions having damaging effects on the function of G6PD protein, whereas, Val107Leu (Score=0.004, Sensitivity=0.97, Specificity=0.59), Asp143Asn (Score=0.063, Sensitivity=0.94, Specificity=0.84) and Ser218Phe (Score=0.018, Sensitivity=0.95, Specificity=0.80) substitutions affecting phenotype to be benign with low scores (Table 3). I-MUTANT predictions showed Ser218Phe (Score=0.018, Sensitivity=0.95, Specificity=0.80) substitution to be deleterious with severe enzyme deficiency (expected accuracy=51%; Table 2).

Table 3. *In silico* prediction analysis for *G6PD* polymorphisms.

SNP	Location	Type	Nucleotide change	Amino acid change	*In silico* prediction results					
					SIFT	PANTHER	SNPs and GO	POLYPHEN	I-MUTANT	MUPRO
rs1050827	Exon2	Non-synonymous	G/C	Gln41His	Deleterious	-2.135	Neutral	Damaging	Neutral	Decrease
rs11555344	Exon4	Non-synonymous	G/T	Val107Leu	Tolerated	-2.95327	Neutral	Benign	Neutral	Decrease
rs5030870	Exon5	Non-synonymous	G/A	Asp143Asn	Tolerated	-3.06584	Disease	Benign	Neutral	Decrease
rs34233392	Exon5	INDEL	-/C	Met155	--	--	--	--	--	--
rs5030868	Exon6	Non-synonymous	C/T	Ser218Phe	Deleterious	-4.73175	Disease	Benign	Deleterious	Increase
rs34193178	Exon9	Non-synonymous	G/C	Asp380His	Deleterious	-4.0413	Disease	Damaging	Neutral	Increase
rs2230036	Exon10	Synonymous	G/A	Gln402Gln	Tolerated	--	--	--	--	--
rs2230037	Exon11	Synonymous	C/T	Tyr467Tyr	Tolerated	--	--	--	--	--
rs1050757	3' UTR	--	A/G	--	--	--	--	--	--	--

SIFT: Sorting Intolerant From Tolerant; **PANTHER**: Protein Analysis THrough Evolutionary Relationships; **SNPs and GO**: Single Nucleotide Polymorphisms and Gene Ontology; **PolyPhen-2**: Polymorphism Phenotyping v2.

While others were predicted to be neutral i.e., Gln41His (expected accuracy=63%), Val107Leu (expected accuracy=83%), Asp143Asn (expected accuracy=83%), and Asp380His (expected accuracy=74%). MUPRO predictions revealed decreased stability of Gln41His (Confidence score= -0.432), Val107Leu (Confidence score= -0.396) and Asp143Asn (Confidence score= -0.771) variants compared to native structure, which may in turn affect the function of the protein. Whereas, Ser218Phe (Confidence score= 0.454) and Asp380His (Confidence score= 0.345) substitutions were shown to increase the stability of G6PD (Table 2). SNPs & GO tool revealed Asp143Asn, Ser218Phe and Asp380His variants show disease like a trait, while Gln41His and Val107Leu were predicted to be neutral (Table 3).

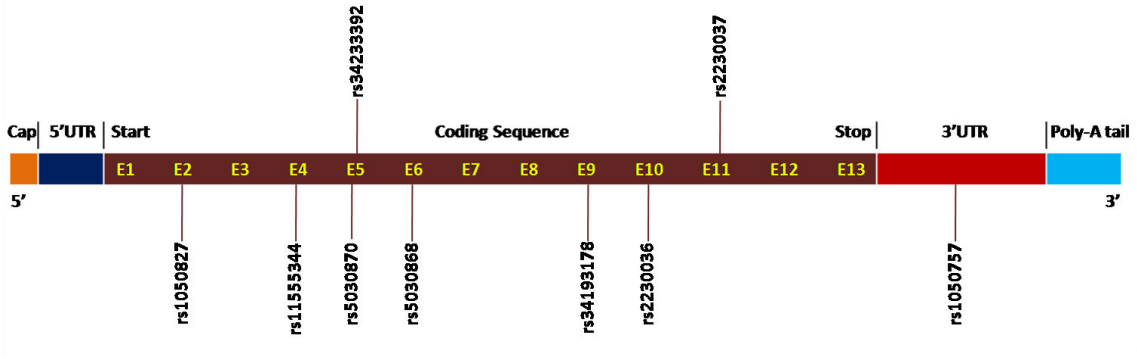

Figure 3. Polymorphisms of *G6PD* gene investigated in the present study.

Table 4: miRNA target prediction: gene target and miRNA seed region in 3' UTR region of target gene.

miRNA	Target gene	Target site in 3' UTR	Predicted consequential pairing of target region (top) and miRNA (bottom)	Seed match
miR-1	*G6PD*	97-103	5' ...AUUGACCUCAGCUGCACAUUCCU... ||||||| 3' UAUGUAUGAAGAAAUGUAAGGU	7mer-m8
		165-171	5' ...UACCCGAGCCCAGCUACAUUCCU... ||||||| 3' UAUGUAUGAAGAAAUGUAAGGU	7mer-m8
		433-439	5' ...CCUCAGUGCCACUUG---ACAUUCCU... |||| ||||||| 3' UAUGUAUGAAGAAAUGUAAGGU	7mer-m8

- 7mer-m8: An exact match to positions 2-8 of the mature miRNA (the seed + position 8).[Source: http://www.targetscan.org/]

4.3.6 miRNA target prediction

Interestingly, our preliminary miRNA target prediction analysis using bioinformatics tools revealed that 3'-UTR of *G6PD* mRNA has 3 binding sites for miR-1. miRNA seed region in 3' UTR region of *G6PD* gene are shown in Table 4.

4.3.7 Genotyping of *G6PD* exon polymorphisms:

The genotyping results demonstrated that all the exons polymorphisms of *G6PD* (rs1050827, rs11555344, rs5030870, rs34233392, rs34193178, rs2230036) except rs5030868 and rs2230037 addressed in the present study, showed only single genotype or allele in both patient and control groups (Figure 4 and 5). However, *G6PD* exon 6 C/T (Ser218Phe; rs5030868; or Mediterranean mutation) and exon 11 C/T (Tyr467Tyr; rs2230037) polymorphisms were not found to be associated with vitiligo, as there was no difference observed in genotype (p=0.7455 and p=0.8013 respectively) as well as allele frequencies (p=0.9278 and p=0.8016 respectively) between vitiligo patients and controls (Table 5). Both control as well as patient populations were under HWE for *G6PD* exon 11 C/T polymorphism (p=0.9434 and p=0.9372 respectively) whereas, both controls and patients were deviated for exon 6 C/T polymorphism (p<0.0001 and p<0.0001 respectively).

4.3.8 Genotyping of *G6PD* 3' UTR A/G (rs1050757) polymorphisms:

The *G6PD* 3' UTR A/G polymorphism was addressed in 366 vitiligo patients and 449 controls where different genotypes were identified using PCR-RFLP (Figure 4). The genotype and allele frequencies differed significantly between patients and controls (p=0.0002 and p=0.0002 respectively). In particular, the minor allele 'G' was more frequent in patients as compared to controls (p=0.0002, OR= 1.488, CI=1.207-1.836; Table 5). Further, 'GG' and 'AG' genotypes were found to be significantly associated with vitiligo (p=0.0142, OR=1.809, CI=1.122-2.919 and p<0.0001, OR=1.792, CI=1.334-2.407 respectively). Both control as well as patient populations were under HWE for *G6PD* 3' UTR polymorphism (p=0.2218 and p=0.1989 respectively).

Further, analysis based on disease progression revealed the increased frequency of genotypes with minor allele 'AG' and 'GG' occurred predominantly in AV patients

compared to controls (p=0.0002 and p=0.0048 respectively; Table 6). Interestingly, the minor allele 'G' was predominant in AV patients compared to controls (p<0.0001) suggesting the important role of minor allele 'G' in disease progression. However, there was no significant difference between AV and SV patients (p=0.268), as well as SV patients and controls (p=0.5807).

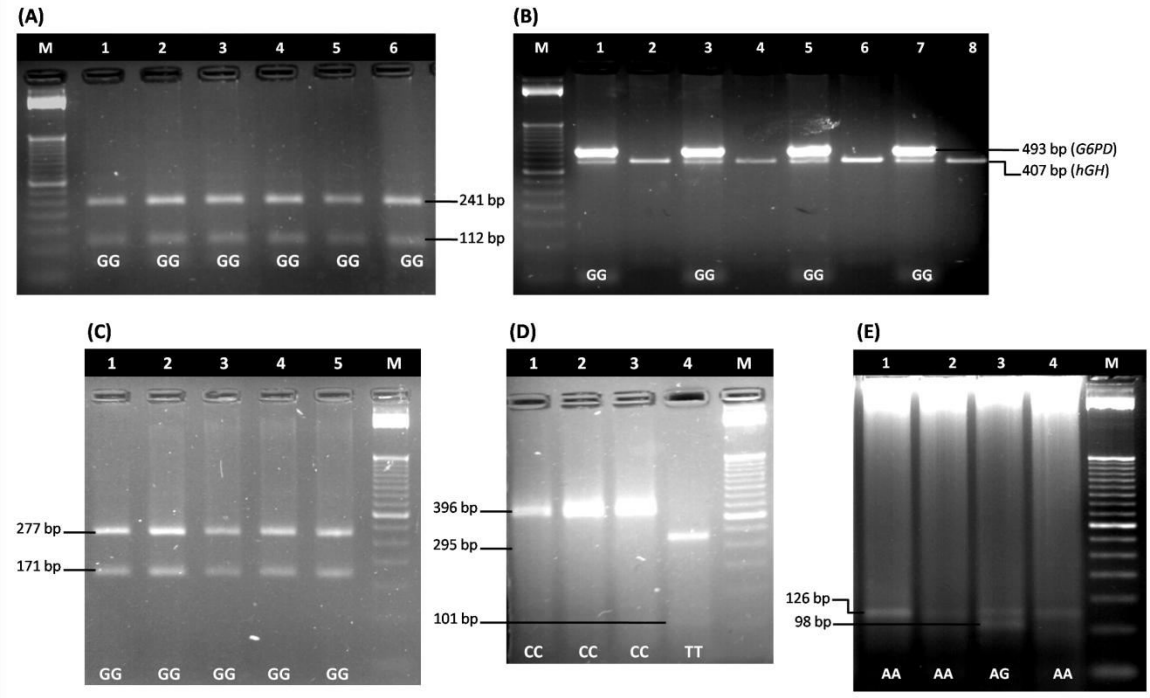

Figure 4. Genotyping of *G6PD* gene polymorphisms: (A) PCR-RFLP analysis of *G6PD* exon 10 G/A (rs2230036) polymorphism on 3.5% agarose gel electrophoresis: lanes 1 to 6 show homozygous (GG) genotypes; lane M shows 50 bp DNA ladder. **(B)** ARMS-PCR analysis of *G6PD* exon 5 G/A (rs5030870) polymorphism on 3.5 % agarose gel electrophoresis: lanes 1 to 8 show homozygous (GG) genotypes; lane M shows 50 bp DNA ladder. **(C)** PCR-RFLP analysis of *G6PD* exon 4 G/T (rs11555344) polymorphism on 3.5% agarose gel electrophoresis: lanes 1 to 5 show homozygous (GG) genotypes; lane M shows 50 bp DNA ladder. **(D)** PCR-RFLP analysis of *G6PD* exon 6 C/T (rs5030868) polymorphism on 3.5 % agarose gel electrophoresis: lanes 1,2 & 3 show homozygous (CC) genotypes; lane 4 shows homozygous (TT) genotype; lane M shows 50 bp DNA ladder. **(E)** PCR-RFLP analysis of *G6PD* 3'UTR A/G (rs1050757) polymorphism on 3.5 % agarose gel electrophoresis: lanes 1,2 & 4 show homozygous

(AA) genotypes; lane 3 shows heterozygous (AG) genotype; lane M shows 50 bp DNA ladder.

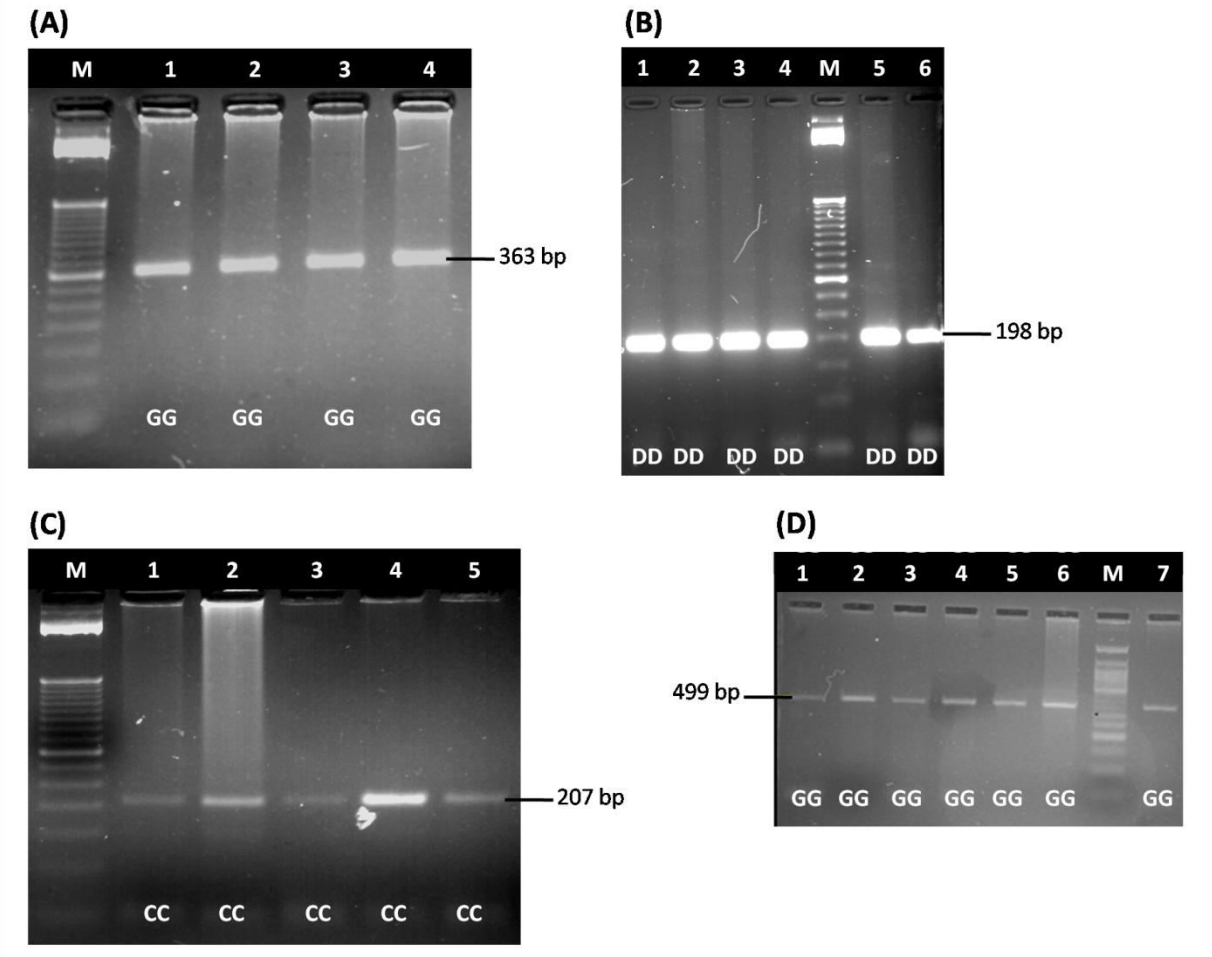

Figure 5. Genotyping of *G6PD* gene polymorphisms: (A) PCR-RFLP analysis of *G6PD* exon 9 G/C (rs34193178) polymorphism on 3.5% agarose gel electrophoresis: lanes 1 to 4 show homozygous (GG) genotypes; lane M shows 50 bp DNA ladder. **(B)** PCR-RFLP analysis of *G6PD* exon 5 -/C (rs34233392) INDEL polymorphism on 3.5 % agarose gel electrophoresis: lanes 1 to 6 show homozygous (DD) genotypes; lane M shows 50 bp DNA ladder. **(C)** PCR-RFLP analysis of *G6PD* exon 11 C/T (rs2230037) polymorphism on 3.5% agarose gel electrophoresis: lanes 1 to 5 show homozygous (CC) genotypes; lane M shows 50 bp DNA ladder. **(D)** PCR-RFLP analysis of *G6PD* exon 2 G/C (rs1050827) polymorphism on 3.5 % agarose gel electrophoresis: lanes 1 to 7 show homozygous (GG) genotypes; lane M shows 50 bp DNA ladder.

Table 5. Distribution of genotype and allele frequencies for *G6PD* polymorphisms in Gujarat vitiligo patients and controls.

SNP	Genotype or Allele	Controls (n=449)	Patients (n=366)	*p* value[#] for Association	OR	95% CI
3'UTR A/G (rs1050757)	AA	239 (0.53)	142 (0.39)	R	1	
	AG	170 (0.38)	181 (0.49)	<0.0001	1.792	1.334-2.407
	GG	40 (0.09)	43 (0.12)	0.0142	1.809	1.122-2.919
	A	648 (0.72)	465 (0.64)	R	1	
	G	250 (0.28)	267 (0.36)	0.0002	1.488	1.207-1.836
Exon 6 C/T (Ser218Phe; rs5030868)	CC	444 (0.99)	361 (0.986)	R	1	
	CT	3 (0.006)	4 (0.011)	0.5149	1.640	0.3645-7.377
	TT	2 (0.004)	1 (0.003)	0.6890	0.6150	0.0555-6.813
	C	891 (0.99)	726 (0.99)	R	1	
	T	7 (0.01)	6 (0.01)	0.9278	1.052	0.3519-3.145
Exon 11 C/T (Tyr467Tyr; rs2230037)	CC	446 (0.993)	363 (0.992)	R	1	
	CT	3 (0.007)	3 (0.008)	0.8013	1.229	0.2464-6.126
	TT	0 (0.0)	0 (0.0)	-	-	-
	C	895 (0.99)	729 (0.99)	R	1	
	T	3 (0.003)	3 (0.004)	0.8016	1.228	0.2470-6.103

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.

Table 6. Distributions of genotype and allele frequencies of *G6PD* 3'UTR polymorphism in different subsets of vitiligo patients and controls.

SNP	Genotype or Allele	Controls (n=449)	AV (n=327)	SV (n=39)	*p* value	GV (n=271)	LV (n=95)	*p* value
3'UTR A/G rs1050757	AA	239 (0.53)	126 (0.38)	16 (0.41)	R	104 (0.38)	38 (0.40)	R
	AG	170 (0.38)	159 (0.49)	22 (0.56)	0.806[a] 0.0002[b] 0.0517[c]	138 (0.51)	43 (0.45)	0.5365[x] 0.0001[y] 0.056[z]
	GG	40 (0.9)	42 (0.13)	1 (0.03)	0.0753[a] 0.0048[b] 0.3272[c]	29 (0.11)	14 (0.15)	0.4587[x] 0.0577[y] 0.0241[z]
	A	648 (0.72)	411 (0.63)	54 (0.69)	R	346 (0.64)	119 (0.63)	R
	G	250 (0.28)	243 (0.37)	24 (0.31)	0.268[a] <0.0001[b] 0.5807[c]	196 (0.36)	71 (0.37)	0.766[x] 0.0009[y] 0.0089[z]

n: number of subjects; R: reference group; AV: Active Vitiligo; SV: Stable Vitiligo; GV: Generalized vitiligo; LV: Localized vitiligo; [#]Vitiligo Patients vs. Controls; [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.

Interestingly, analysis based on type of vitiligo shown the increased frequency of genotypes with minor allele 'AG' in GV patients and 'GG' in LV patients compared to controls (p=0.0001 and p=0.0241 respectively; Table 6), suggesting the important role of 'G' allele in LV. The minor allele 'G' was predominant in GV as well as LV patients compared to controls (p=0.0009 and p=0.0089 respectively). However, there was no significant difference between GV and LV patients (p=0.766).

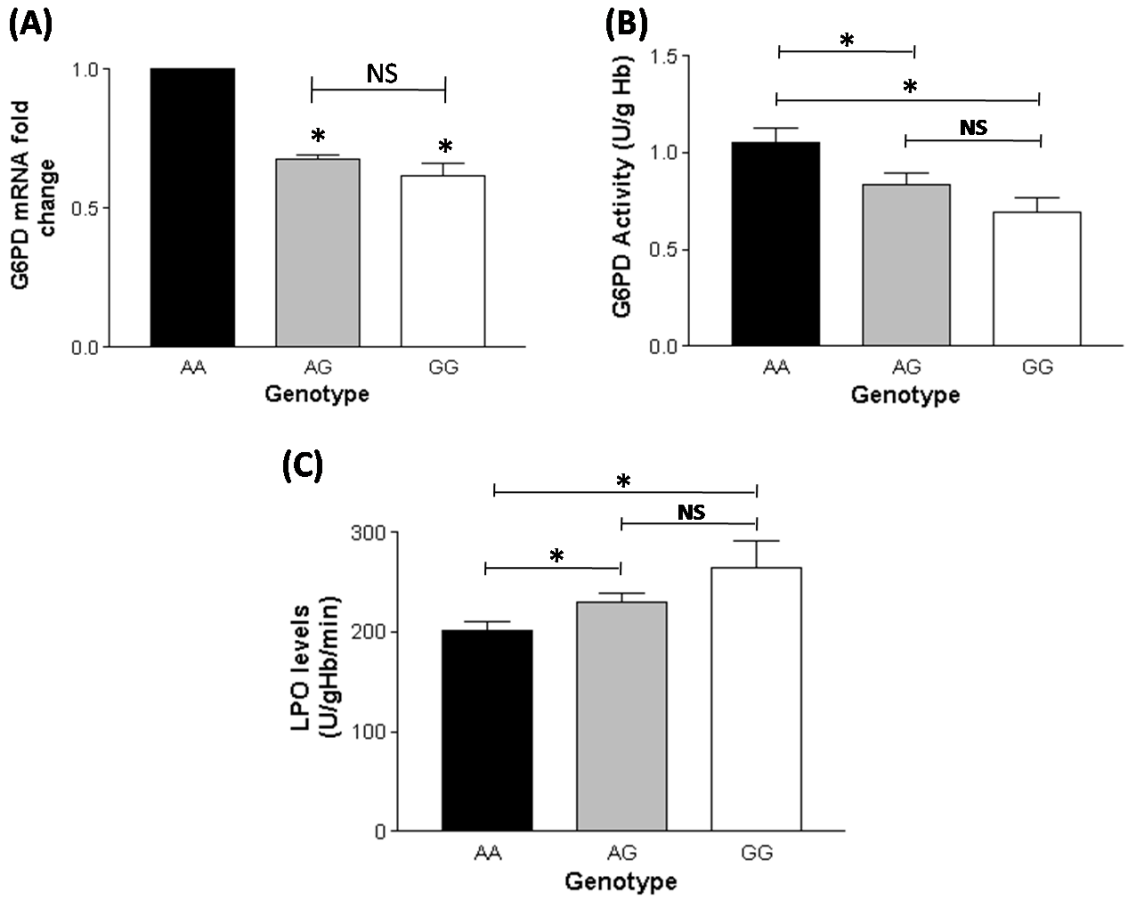

Figure 6: Genotype -phenotype correlation analysis for *G6PD* 3' UTR A/G polymorphism: **(A)** Individuals with GG and GA genotypes showed significantly decreased *G6PD* transcript levels as compared to individuals with AA genotype (p= 0.0266 and p=0.0281 respectively). **(B)** Individuals with GG and AG genotypes showed significantly decreased G6PD activity compared to individuals with AA genotype (p=0.0176 and p=0.0492 respectively). **(C)** Individuals with GG and AG genotypes showed significantly increased LPO levels as compared to individuals with AA genotype (p=0.0159 and p=0.0339 respectively). [*p<0.05; NS: non-significant (p>0.05)].

4.3.9 Genotype -phenotype correlation analysis for *G6PD* 3' UTR A/G polymorphism:

G6PD mRNA, its activity and LPO levels were analyzed with respect to *G6PD* 3' UTR A/G genotypes: individuals with GG and GA genotypes showed significantly decreased

G6PD mRNA levels as compared to individuals with AA genotype (Mean ΔCt ± SEM: 5.972±0.325 and 5.828±0.2029 vs. 5.268±0.1478; p= 0.0266 and p=0.0281 respectively; Figure 6A). Further, individuals with GG and AG genotypes showed significantly decreased G6PD activity compared to individuals with AA genotype (Mean ± SEM: 0.6926 ± 0.07829 and 0.8343 ± 0.06452 vs. 1.052 ± 0.07764; p= 0.0176 and p=0.0492 respectively; Figure 6B). However, there was no significant difference for G6PD mRNA (p=0.706) as well as activity (p=0.196) between individuals with AG genotype and GG genotype. In addition, individuals with GG and AG genotypes showed significantly increased LPO levels as compared to individuals with AA genotype (Mean ± SEM: 264.9 ± 27.13 and 229.7 ± 10.04 vs. 201.2 ± 8.887; p= 0.0159 and p= 0.0339 respectively; Figure 6C). However, there was no significant difference for LPO levels between individuals with AG genotype and GG genotype (p= 0.2036).

4.4 DISCUSSION

A single dominant pathway is implausible to account for all cases of melanocyte loss in vitiligo; rather, it is the result of complex interactions of biochemical, environmental and immunological events, in a permissive genetic milieu (Shajil *et al*., 2006; Laddha *et al*., 2013a; Mansuri *et al*., 2014c; Dwivedi *et al*., 2015). The auto-cytotoxic premise suggests that oxidative stress is one of the major players in melanocyte destruction and is believed to be the initial triggering event of vitiligo, with subsequent accumulation of H_2O_2 in the epidermis of patients (Schallreuter *et al*., 1999; Bickers and Athar, 2006; Spencer *et al*., 2007; Namazi, 2007). It has been reported that inducers of hemolysis like anti-malarials and henna, in G6PD-deficient individuals has the potential to exacerbate vitiligo through generating free radicals that exhaust the G6PD-dependant detoxifying enzymes (Roth *et al*., 1960). In our recent study, we have observed reduced G6PD activity and higher LPO levels resulting in decreased viability of SK-MEL cells upon treatment with 6-amino nicotinamide (6-ANAD), a G6PD inhibitor (Unpublished data). The present study showed decreased *G6PD* mRNA as well as activity (Figure 2) and increased LPO levels, which are in accordance with previously reported reduced G6PD activity and increased LPO levels in vitiligo patients (Agrawal *et al*., 2004; Shajil and Begum, 2006; Arican and Kurutas, 2008; Farahi-Jahromy *et al*., 2014), supporting the oxidative stress hypothesis of

vitiligo. Under oxidative stress, miR-1 expression was reported to be high and favored apoptosis (Xu *et al*., 2007). We have earlier reported increased miR-1 expression in the lesional skin of patients with vitiligo (Mansuri *et al*., 2014b). In the present study, miR-1 showed increased expression in the whole blood of patients especially with AV and GV, as compared to controls (Figure 1). Interestingly, miR-1 targets *G6PD* (Xu *et al*., 2007; Singh *et al*. 2013), anti-apoptotic HSP60 and HSP70, which inhibit the mitochondrial death pathway in the cell (Vecchione and Croce, 2010). In agreement to these, the present study showed down-regulation of *G6PD* in lesional skin of patients (Figure 2A) as well as in blood circulation of active-generalized vitiligo patients (Figure 6), supporting miR-1 mediated *G6PD* expression and its involvement in vitiligo progression.

G6PD (EC 1.1.1.49) is a dimer and each subunit is of 59.3 kDa. The human *G6PD* gene is located on human chromosome Xq28. It spans 16 kb and consists of 13 exons and 12 introns. Haplotype analysis on the 12 segregating sites located between exons 3 and 13 of G6PD showed that only two haplotypes were modal across all populations, indicating low haplotype diversity (Sarkar *et al*., 2010). These two haplotypes ('CTA' & 'TCG') differed only at the polymorphic sites rs2230037 (C/T), rs2071429 (T/C) and rs1050757 (A/G), and possessed by ~80% of studied individuals from 10 ethnic groups of India. It has been suggested that the severely restricted number of haplotypes and low haplotype diversity are likely due to strong natural selection (Kim *et al*., 2014). Studying the haplotypes created by combinations of these polymorphisms may be useful in identifying the molecular basis of G6PD deficiency in vitiligo. We performed sequence based analysis to predict the impact of *G6PD* exonic polymorphisms on G6PD protein using *in silico* prediction tools (Table 3). Our results revealed decreased stability of G6PD variants compared to native protein, which might in turn affect the function of the protein and show disease like a trait (Table 3). The polymorphisms studied in the present study are shown in Figure 3. However, the genotyping results demonstrated that all the exonic polymorphisms of *G6PD* (rs1050827, rs11555344, rs5030870, rs34233392, rs34193178, rs2230036) except rs5030868 and rs2230037; addressed in the present study showed only single genotype or allele in both patient and control groups, suggesting highly conserved loci of *G6PD* in Gujarat population. In support to our results, *G6PD* exon 9 G/C (rs34193178) polymorphism was also reported to be monomorphic in the Korean

population (Kim *et al*., 2014). *G6PD* exon 10 G/A (rs2230036) polymorphism was reported to be associated with increased susceptibility to malaria in Fulani males (Maiga *et al*., 2014). The polymorphic *G6PD* variants in each population have a characteristic profile (Minucci *et al*., 2012). The *G6PD* Mediterranean variant (exon 6 C/T SNP) is predominant in the Mediterranean and Middle East regions (Kurdi-Haidar *et al*., 1990). Beutler and Kuhl (1990) have mentioned that *G6PD* Mediterranean mutation may have originated independently in Europe and Asia. *G6PD* exon 11 C/T (rs2230037) synonymous coding sequence polymorphism (also called as *G6PD* 1311 C/T polymorphism) was found to be polymorphic in different populations from Middle East (Kurdi-Haidar *et al*., 1990), Africa (Vulliamy *et al*., 1991), Europe, Asia (Beutler and Kuhl, 1990), including India (Sarkar *et al*., 2010) and Afghanistan (Jamornthanyawat *et al*., 2014) and Korea (Kim *et al*., 2014). *G6PD* Viangchan (871G/A, 1311C/T) is the most common mutation observed in Phuan tribe of Thailand, where G6PD deficiency is highly frequent (Cheepsunthorna and Nuchprayoon, 2013). On contrary, it has also been reported that this marker is of no functional significance (Jamornthanyawat *et al*., 2014). Recently, Dewasurendra *et al.* (2015) have reported that *G6PD* exon 11 C/T SNP had the ancestral allele as the major allele in Sri Lankan population. Our results are in accordance to Korean (Kim *et al*., 2014) and Sri Lankan (Dewasurendra *et al*., 2015) populations for G6PD exonic polymorphisms.

On the other hand, 3'UTR (rs1050757) polymorphism was found to be associated with vitiligo patients in the present study (Table 5); where minor allele 'G' was predominantly present in active-generalized/ localized vitiligo patients as compared to controls (Table3). The minor allele containing genotypes 'AG' and 'GG' were predominantly present in active vitiligo patients as compared to controls (Table3). Interestingly, 'AG' genotype was more frequently present in active vitiligo, whereas 'GG' genotype was more frequently present in localized vitiligo as compared to controls (Table3). These results suggest the crucial role of minor allele 'G' of 3'-UTR polymorphism in development and progression of vitiligo. Previously, a strong association was observed between *G6PD* 3'UTR rs1050757G and 1311T in Malaysian aboriginal group, Negrito (Amini and Ismail, 2013).

The 3'UTR of human *G6PD* gene has two AG-rich regions (with 17 and 35 nucleotides length) and rs1050757 is located in the 35 bp AG-rich region (Amini and Ismail, 2013). The importance of nucleotide-rich elements (AU-, C-, CU- and AG rich element) in 3'UTR have been implicated in mRNA stability through changes in secondary structure of mRNA (Wang *et al*., 2001; Knowles *et al*., 2003; Peyvandi *et al*., 2005). Amini and Ismail (2013) have reported that rs1050757G caused significant alterations in the secondary structure of mutant *G6PD* transcript.

Furthermore, it has been reported that a majority of non- synonymous mutations also cause some degree of alteration to the mRNA structure (Johnson *et al*., 2011). In contrast, growing number of evidence suggest that mRNA structural rearrangement might be considered as a proposed mechanism for disease-associated mutations (Halvorsen *et al*., 2010). Also, it is believed that structural conformation of mRNA influences gene expression and regulation. Likewise, it has been demonstrated that synonymous mutations in coding regions led to differential mRNA folding structure of human replication Protein A and human Alanyl tRNA synthetase (Shen *et al*., 1999). Amini and Ismail (2013) proposed that the new conformation of mRNA placed some sequences in the open structures such as loop or arc; therefore, these sequences are no longer involved in the base pairing and they are accessible to post-transcriptional modulators such as miRNA, to explain G6PD deficiency in haplotype of 1311T/93C/rs1050757G. It was assumed that detection of the allele 1311T alone, which neither changes amino-acid sequence nor mRNA secondary structure, is a neutral mutation probably due to its position in loop structure. However, the combination of mutant alleles 1311T and rs1050757G caused significant alterations in the secondary structure of *G6PD* mRNA. In other words, in the secondary structure of 3'UTR with normal allele rs1050757A, a possible standard Watson–Crick paired duplex was observed in the majority of predicted miRNA binding sites. However, mutant allele G shows reshuffling of the base pairings resulting in a different predicted secondary structure in some regions. Further, a total 152 putative target sites were predicted for 118 miRNAs in normal 3'UTR using bioinformatics programs (Amini and Ismail, 2013). Interestingly, transition of A to G creates two more binding sites for hsa-miR-1238 and hsa-miR-877* where, rs1050757G is located inside the ''seed region'' of these miRNAs. However, further analysis revealed

that in normal mRNA only 1 out of 152 potential target sites are accessible to the three miRNAs (hsa-miR-206, hsa-miR-613 and hsa-miR-1) owing to the mRNA folding. Moreover, transition of A to G at position rs1050757 leads to accessibility of two more binding sites for hsa-miR-3173 and hsa-miR-149*. Amini and Ismail (2013) postulated that miRNA target site was not accessible in the wild type mRNA because of its secondary structure, but in the mutant mRNA the binding sites were accessible to miRNA(s) for perfect complimentary of seed region. Notably, a number of miRNAs can induce mRNA degradation as well as translational repression, whereas the others act primarily on translation (Lee and Shatkay, 2008). Consistent with these data, in the present study the genotype –phenotype correlation analysis for 3'UTR rs1050757 polymorphism revealed that individuals with GG and GA genotypes exhibit significantly decreased *G6PD* transcript levels as well as decreased activity, as compared to individuals with AA genotype (Figure 6A & 3B), suggesting the important role of 3'-UTR A/G polymorphism in oxidative stress mediated pathogenesis of vitiligo. This was further supported by genotype-phenotype correlation analysis with respect to LPO levels which is an indicator of oxidative stress; where individuals with GG and GA genotypes exhibit significantly increased LPO levels as compared to individuals with AA genotype (Figure 6C). This is the first report of biochemical association of a polymorphism in 3'UTR of *G6PD* gene and the possible role of miRNA in its regulation.

In conclusion, we confirm for the first time the genetic association of vitiligo with *G6PD* polymorphisms, which contains at least two independent risk factors, one tagged by 3'UTR rs1050757 polymorphism of *G6PD* and another tagged by miR-1 regulated *G6PD* expression in vitiligo patients. We have found these novel factors could contribute to oxidative stress mediated pathogenesis of vitiligo.

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