CHAPETR VI

MELANOCYTE PROLIFERATING GENE 1 (*MYG1*) PROMOTER AND STRUCTURAL POLYMORPHISMS AND THEIR GENOTYPE-PHENOTYPE CORRELATION WITH VITILIGO SUSCEPTIBILITY

6.1 INTRODUCTION

Vitiligo is an acquired hypomelanotic disease characterized by circumscribed depigmented macules. Absence of melanocytes from the lesional skin due to their destruction has been suggested to be the key event in the pathogenesis of vitiligo (Ortonne and Bose, 1999). It is frequently associated with a positive family history and affects approximately 0.5-1% of the world population (Taieb, 2007). In India, the incidence of vitiligo is found to be 0.5-2.5% wherein Gujarat and Rajasthan states have the highest prevalence upto ~8.8% (Handa and Kaur, 1999). Autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity (Shajil *et al.*, 2006; Kemp *et al.*, 2001). The autoimmune hypothesis gains further support from immunotherapy studies of melanoma patients (Rosenberg, 1997).

Vitiligo is a polygenic disease and attempts to identify genes involved in susceptibility include gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses to discover new genes. Recent genome-wide association studies (GWAS), have identified generalized vitiligo susceptibility genes which involve immune regulation and immune targeting of melanocytes, suggesting that generalized vitiligo is a primary autoimmune disease however, the biological triggers of the autoimmune process remain unknown (Spritz, 2011). Several candidate genes have been tested for genetic association with generalized vitiligo, including the *MHC*, *ACE*, *CAT*, *CTLA*-4, *COMT*, *ESR*, *GCH*1, *MBL2*, *PTPN*22, and *VDR* (Spritz, 2007 & 2008). Most of these studies reported significant associations, although some yielded only marginal significance and several were not replicated by subsequent studies. Recently, a number of genes which play a role in vitiligo susceptibility, including *HLA*, *PTPN*22, *NALP*1, *XBP*1, *FOXP*1, *IL2RA* have been tested for genetic association with vitiligo (Spritz, 2010). *MYG1* [Melanocyte proliferating gene 1, C12orf10 in human] is a recently described gene of unknown function. According to one study *MYG1* is a highly expressed gene in freely proliferating melanocytes and down regulated in malignant melanoma cells and suggesting that *MYG1* could be a melanocyte specific gene (Smicun, 2000).

MYG1 is a ubiquitous nucleo-mitochondrial protein involved in early developmental processes (Philips *et al.*,2009); however *MYG1* expression in normal adult tissues is stable and seems to be changed mainly as a response to stress/illness conditions (Kingo *et al.*,2006; Hawse *et al.*, 2003; Kõks *et al.*,2004). MYG1 is the only member of an uncharacterized protein family UPF0160 (PF03690) and contains a number of metal binding residues and is predicted to possess metal-dependent protein hydrolase activity. MYG1 protein is highly conserved and present in all eukaryotes from yeast to humans (Nepomuceno-Silva *et al.*, 2004).

According to global expression analysis, *MYG1* tends to be up-regulated in undifferentiated and pluripotent cells. Kingo *et al.*, (2006) have suggested the involvement of *MYG1* in vitiligo pathogenesis by showing elevated expression of *MYG1* mRNA in both uninvolved and involved skin of vitiligo patients. In addition, *MYG1* has been found to be consistently up-regulated in skin biopsies from patients with atopic eczema (Sääf *et al.*, 2008).

Recently, Philips *et al.*, (2010) have proposed that *MYG1* gene may be involved in vitiligo pathogenesis. This study demonstrated that both *MYG1* promoter polymorphism -119C/G and Arg4Gln polymorphism in the mitochondrial signal of *MYG1* have a functional impact on the regulation of the *MYG1* gene and promoter polymorphism (-119C/G) is related with susceptibility for actively progressing vitiligo in Caucasian population. Hence, the study has prompted us to investigate the involvement of *MYG1* gene in precipitation of vitiligo in Gujarat population.

The objectives of this study were:

i.) To determine whether *MYG1* promoter (rs1465073) and structural (rs1534284-rs1534283) polymorphisms are associated with vitiligo susceptibility in Gujarat population.

ii.) To measure and compare *MYG1* transcript levels in patients with vitiligo and in unaffected controls.

iii.) To determine genotype-phenotype correlation of *MYG1* with vitiligo susceptibility.

6.2 MATERIALS AND METHODS

6.2.1 Study subjects

The study group included 846 vitiligo patients [668 generalized (including acrofacial vitiligo and vitiligo universalis) and 178 localized vitiligo cases] comprised of 389 males and 457 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune disease. A total of 726 ethnically sex-matched unaffected individuals (347 males and 379 females) were included as controls in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

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. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls

	Vitiligo Patients	**Controls**
	(n = 846)	(n = 726)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	389 (45.98%)	347 (47.79%)
Female	457 (54.02%)	379 (52.21%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Type of vitiligo		
Generalized	668 (78.96%)	NA
Localized	178 (21.04%)	NA
Active vitiligo	599 (70.80%)	NA
Stable vitiligo	247 (29.20%)	NA
Family history	116 (13.71%)	NA

6.2.2 Genomic DNA Preparation

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA). Genomic DNA was extracted from whole blood using 'QIAamp DNA Blood Kit' (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

6.2.3 Determination of *MYG1* promoter and structural polymorphisms

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype promoter and structural polymorphisms of *MYG1* gene (Figure 1A & B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes (New England Biolabs, Beverly, MA) *Hinf*I and *Tas*I were used for digesting amplicons of -119C/G and Arg4Gln (AA/GC) respectively of *MYG1* gene (Table 2). 5 µL of the amplified products were digested with 5 U of the corresponding restriction enzyme in a total reaction volume of 25 µL, according to the manufacturer's instructions. The digested products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved on 2.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were

100% concordant (analysis of the chosen samples was repeated by two researchers independently).

Table 2. Primers and restriction enzymes used for *MYG1* SNPs genotyping and gene expression analyses.

Gene/SNP	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs1465073)				
MYG1 -119C/G F	GGATTAAGCAAGGGACTGGTGATG	60	347	*Hinf*I (150 & 197 bp)
MYG1 -119C/G R	CATGCGGTGCCGGGTATACA			
(rs1534284-rs1534283)				
MYG1 AA/GC F	GGATTAAGCAAGGGACTGGTGATG	63	347	*Tas*I (282 & 65 bp)
MYG1 AA/GC R	CATGCGGTGCCGGGTATACA			
MYG1 expression F	GGAGTACCGGGATGCAGAG	65	290	-
MYG1 expressionR	CATACATCTTGTCATAGAGGGTGC			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expressionR	CAAATGAGCCCCAGCCTTCT			

6.2.4 Determination of *MYG1* and *GAPDH* mRNA expression

6.2.4.1 RNA extraction and cDNA synthesis

Total RNA from whole blood was isolated and purified using Ribopure™- blood Kit (Ambion inc. Texas, USA) following the manufacturer's protocol. RNA integrity was

verified by 1.5% agarose gel electrophoresis, RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentase, Vilnius, Lithuania) according to the manufacturer's instructions in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

6.2.4.2 Real-time PCR

The expression of *MYG1* and *GAPDH* transcripts were measured by real-time PCR using SYBR Green method 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 (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 10 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 3E). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

6.2.5 Statistical analyses

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for both the polymorphisms in patients and controls by comparing the observed and expected frequencies of genotypes using chi-squared analysis. Distribution of the genotypes and allele frequencies of -119C/G and Arg4Gln (AA/GC) polymorphisms for patients and control subjects were compared using the chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. *p*-values less than 0.025 were considered as statistically significant due to Bonferroni's correction for multiple

testing. Age of onset analysis and relative expression of *MYG1* in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). The statistical power of detection of the association with the disease at the 0.05 level of significance was determined by using the G* Power software (Faul *et al.*, 2007).

6.3 RESULTS

6.3.1 Analysis of association between *MYG1* -119C/G polymorphism and susceptibility to vitiligo

PCR-RFLP for *MYG1* -119C/G polymorphism yielded a 347 bp undigested product corresponding to G allele and 197 bp and 150 bp digested products corresponding to C allele. The three genotypes identified by 2.5% agarose gel electrophoresis were: CC homozygous, CG heterozygous and GG homozygous for -119C/G polymorphism of *MYG1* gene (Figure 1A).

The genotype and allele frequencies of the -119C/G polymorphism in 846 vitiligo patients and 726 controls are summarized in Table 3. The *MYG1* -119C/G polymorphism was found to be in significant association with vitiligo patients ($p<0.0001$) when genotypes were compared using chi-squared test-3x2 contingency table (Table 3). The minor allele (G) of the -119C/G was more frequent in the vitiligo group compared to the control group (65.0% versus 57.0%, $p<0.0001$; OR 1.405, 95% CI 1.216 - 1.623) consistent with a susceptibility effect (Table 3). Control population was found to be in Hardy-Weinberg equilibrium for this polymorphism however, patient population deviated from the equilibrium ($p=0.079$ and $p=0.018$ respectively) (Table 3). Moreover, both generalized and localized vitiligo groups showed significant association of -119C/G polymorphism when the genotypes were compared with those of control group ($p=0.0003$ and $p=0.006$ respectively) (Table 4). Also, there was significant difference in allele frequencies of the polymorphism for generalized and localized vitiligo groups when the alleles were compared with those of control group ($p<0.0001$ and $p=0.002$ respectively) (Table 4). The distribution of *MYG1* -119C/G genotype frequencies was consistent with Hardy-Weinberg expectations in control as well as both the patient groups ($p>0{\cdot}05$).

In addition, gender based analysis of *MYG1* -119C/G polymorphism suggested significant association of minor -119G allele, consistent with a susceptibility effect with female vitligo patients as compared to male patients (69.0% versus 60.0%, p=0.0003, OR 1.454, 95% CI 1.190-1.777 (Table 5). This study has 93.77% statistical power for the effect size 0.08 to detect the association of investigated polymorphisms at $p<0.05$ in generalized vitiligo patients and control population.

6.3.2 Analysis of association between *MYG1* Arg4Gln (AA/GC) polymorphism and susceptibility to vitiligo

The genotype and allele frequencies of the investigated *MYG1* Arg4Gln (AA/GC) polymorphism in 846 vitiligo patients and 726 controls are summarized in Table 3. PCR-RFLP for *MYG1* Arg4Gln (AA/GC) polymorphism yielded only a 347 bp undigested product corresponding to GC alleles, identified by 2.5% agarose gel electrophoresis (Figure 1B).

The *MYG1* Arg4Gln (AA/GC) polymorphism was not found to be associated with vitiligo susceptibility ($p=1.00$) (Table 3). Interestingly, the polymorphism was found to be monogenic; being only GC alleles presented in control and both the patient groups Table 3. Also, the distribution of genotype frequencies for *MYG1* promoter polymorphism was not consistent with Hardy-Weinberg expectations in both patient and control groups ($p<0.0001$).

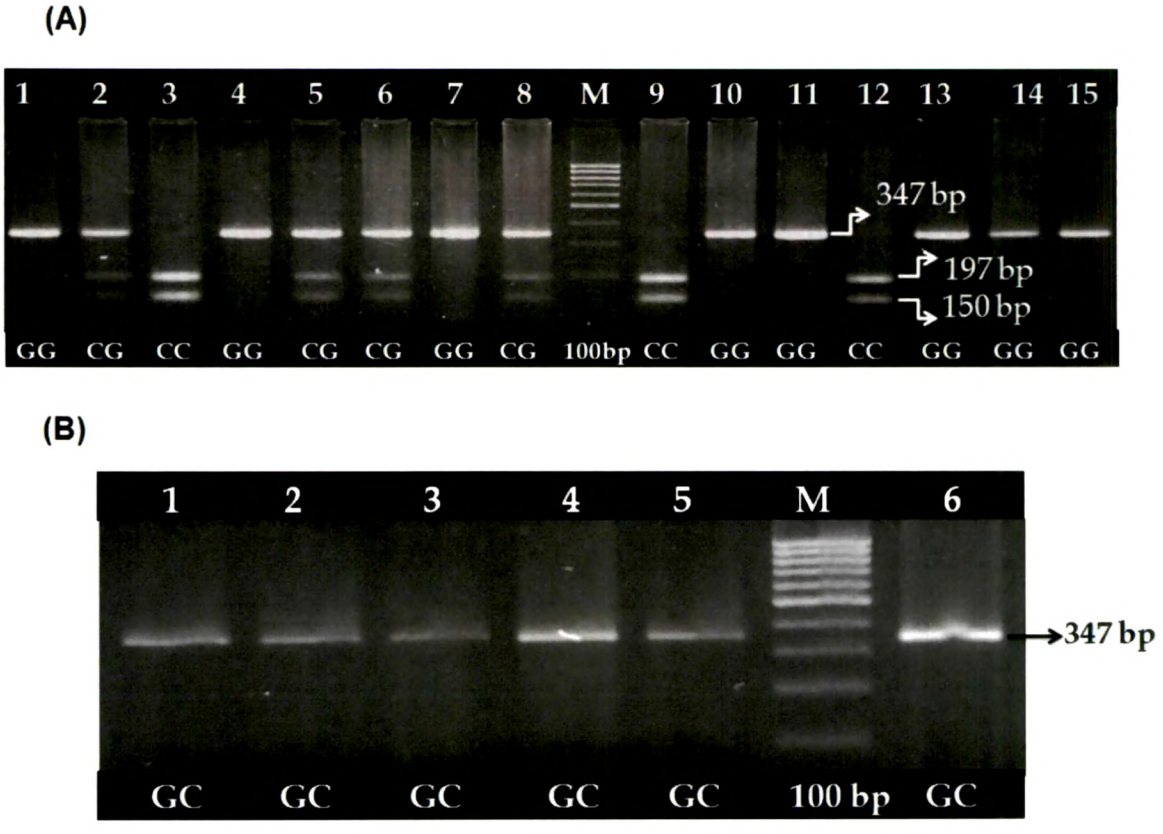

Figure 1. PCR-RFLP analysis of *MYG1* -119C/G and 11-12 AA/GC polymorphisms:

(A) PCR-RFLP analysis of *MYG1* -119C/G polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 4, 7, 10, 11, 13, 14 &15 show homozygous (GG) genotypes; lanes: 2, 5, 6, & 8 show heterozygous (CG) genotypes; lanes: 3, 9 & 12 show homozygous (CC) genotypes; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *MYG1* 11-12 AA/GC polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 2, 3, 4, 5 & 6 show individuals with GC alleles; lane M shows 100 bp DNA ladder.

Table 3. Association studies for *MYG1* gene -119C/G promoter and 11-12AA/GC structural polymorphisms in vitiligo patients from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 846)	(n = 726)			
	CC	120 (0.14)	147 (0.20)		0.018[a]	
rs1465073	CG	354 (0.42)	333 (0.46)	<0.0001	(P)	
Promoter	GG	372 (0.44)	246 (0.34)			
(-119C/G)	Allele				0.079[b]	1.405
	C	594 (0.35)	627 (0.43)	<0.0001	(C)	(1.216-
	G	1098 (0.65)	825 (0.57)			1.623)
	Genotype	(n = 846)	(n = 726)			
	AA	0 (0.00)	0 (0.00)		<0.0001[a]	
rs1534284-	AG	0 (0.00)	0 (0.00)	1.000	(P)	
rs1534283	GC	846 (1.00)	726 (1.00)			
	Allele					1.000
(AA/GC;	G	423 (0.50)	363 (0.50)	1.000	<0.0001[b]	(0.801-
	C	423 (0.50)	363 (0.50)			1.235)
Arg4Gln)					(C)	

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval,

(P) refers to Patients and (C) refers to Controls,

[a] Vitiligo Patients vs. Controls using chi-squared test with 3 × 2 contingency table,

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

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

Table 4. Association studies for *MYG1* gene -119C/G promoter and 11-12AA/GC structural polymorphisms in generalized and localized vitiligo patients from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo Patients (Freq.)	Localized Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 668)	(n = 178)	(n = 726)			0.7212
	CC	94 (0.14)	26 (0.15)	147 (0.20)	0.0003[a]	0.082	(0.6189-
rs1465073	CG	285 (0.43)	69 (0.39)	333 (0.46)		(GV)	0.8403)
Promoter	GG	289 (0.43)	83 (0.46)	246 (0.34)	0.006[b]		(GV)
(-119C/G)	Allele					0.069	
	C	473 (0.35)	121(0.34)	627 (0.43)	<0.0001[c]	(LV)	0.6818
	G	863 (0.65)	235(0.66)	825 (0.57)			(0.5349-
					0.002[d]	0.079	0.8692)
						(C)	(LV)
	Genotype	(n = 668)	(n = 178)	(n = 726)			
	AA	0 (0.00)	0 (0.00)	0 (0.00)		<0.00	
rs1534284-	AG	0 (0.00)	0 (0.00)	0 (0.00)	1.000	01	1.000
rs1534283	GC	668 (1.00)	178(1.00)	726 (1.00)	(GV, LV)	(GV,	(0.801-
(AA/GC;	Allele					LV)	1.235)
Arg4Gln)	G	334 (0.50)	89 (0.50)	363 (0.50)	1.000		(GV,
	C	334 (0.50)	89 (0.50)	363 (0.50)	(GV, LV)	<0.00	LV)
						01	
						(C)	

'n' represents number of Patients/ Controls,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval,
(GV) refers to Generalized Vitiligo, (LV) Localized Vitiligo and (C) refers to Controls,
[a] Generalized Vitiligo vs. Controls using chi-squared test with 3×2 contingency table,
[b]Localized Vitiligo vs. Controls using chi-squared test with 3×2 contingency table,
[c] Generalized Vitiligo vs. Controls using chi-squared test with 2×2 contingency table,
[d] Localized Vitiligo vs. Controls using chi-squared test with 2 × 2 contingency table,
Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

Table 5. Association studies for *MYG1* gene -119C/G promoter and 11-12AA/GC structural polymorphisms in male and female patients with vitiligo.

SNP	Genotype or allele	Male Patients (Freq.)	Female Patients (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 389)	(n = 457)			
	CC	70 (0.18)	50 (0.11)		0.067	
rs1465073	CG	169 (0.43)	185 (0.40)	0.002[a]	(M)	
Promoter	GG	150 (0.39)	222 (0.49)			
(-119C/G)	Allele				0.225	1.454
	C	309 (0.40)	285 (0.31)	0.0003[b]	(F)	(1.190-
	G	469 (0.60)	629 (0.69)			1.777)
	Genotype	(n = 389)	(n = 457)			
	AA	0 (0.00)	0 (0.00)		<0.0001	
	AG	0 (0.00)	0 (0.00)	1.000	(M)	1.000
rs1534284-	GC	389 (1.00)	457 (1.00)			(0.801-
rs1534283	Allele				<0.0001	1.235)
(AA/GC;	G	389 (0.50)	457 (0.50)	1.000	(F)	
Arg4Gln)	C	389 (0.50)	457 (0.50)			

'n' represents number of Patients,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval,
(M) refers to Male Patients and (F) refers to Female Patients,
[a] Male Patients vs. Female Patients using chi-squared test with 3×2 contingency table,
[b]Male Patients vs. Female Patients using chi-squared test with 2×2 contingency table,
Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

6.3.3 Effect of *MYG1* -119C/G genotypes on age of onset of vitiligo and its progression

When age of onset of the disease was correlated with the *MYG1* -119C/G genotypes, patients with susceptible GG genotypes showed early onset of the disease as compared to CC and CG genotypes (p=0.0002 and p=0.039 respectively) (Figure 2A). Moreover, patients with genotype CG showed early onset of the disease as compared to CC genotypes (p=0.022) (Figure 2A) suggesting the effect of the susceptible allele G on the early onset of disease. Interestingly, when male and female vitiligo patients were analyzed for age of onset of the disease, female patients had significant early onset of the disease as compared to the male patients (p=0.002) (Figure 2B).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the minor -119G allele occurred prevalently in the group of patients with active vitiligo (68.0% versus 57.0%, p<0.0001; OR 0.6207, 95% CI 0.5291-0.7282) consistent with a susceptibility effect compared to the control group (Table 6). However, there was no statistically significant effect in the distribution of the -119G allele between patients with stable vitiligo and control group (p=0.833) (Table 6). Interestingly, the -119G allele was more prevalent in patients with active vitiligo as compared to stable vitiligo (68.0% versus 57.0%, p<0.0001; OR 0.6380, 95% CI 0.5141-0.7916) suggesting the important role of -119G allele in progression of the disease (Table 6).

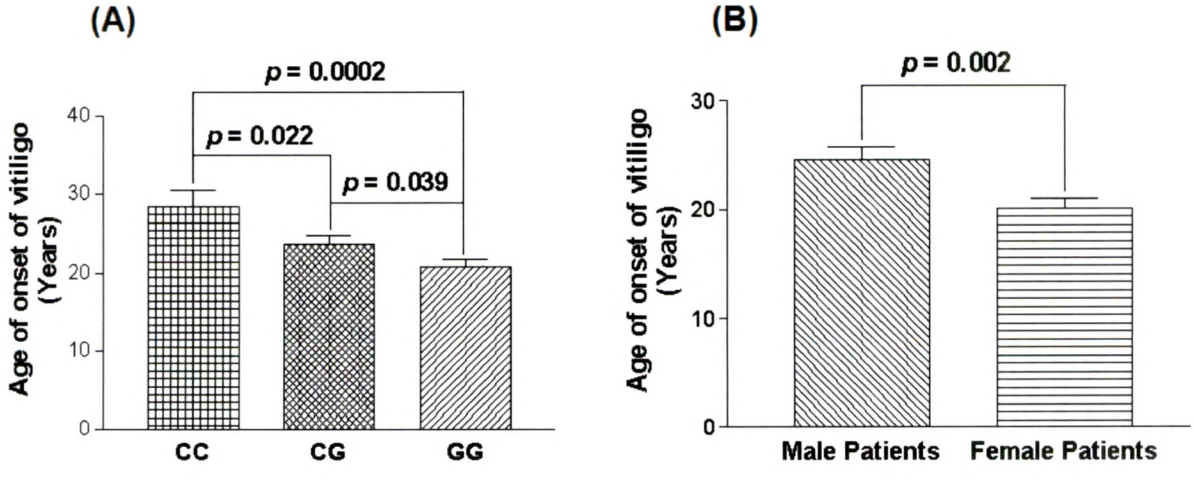

Figure 2. Age of onset of the disease in vitiligo patients:

(A) Comparison of age of onset of the disease (Years) with respect to *MYG1* promoter -119C/G in 846 vitiligo patients.

(B) Comparison of age of onset of the disease (Years) with respect to gender differences in 389 male patients and 457 female patients with vitiligo.

Table 6. Association studies for *MYG1* gene -119C/G promoter and 11-12AA/GC structural polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 599)	(n = 247)	(n = 726)			0.6380[a]
	CC	69 (0.13)	51 (0.17)	147 (0.20)	0.0003[a]	0.162	(0.5141-
rs1465073	CG	246 (0.42)	108 (0.42)	333 (0.46)	<0.0001[b]	(AV)	0.7916)
Promoter	GG	284 (0.45)	88 (0.41)	246 (0.34)	0.833[c]		
(-119C/G)	Allele					0.098	0.6207[b]
	C	384 (0.32)	210 (0.43)	627 (0.43)	<0.0001[a]	(SV)	(0.5291-
	G	814 (0.68)	284 (0.57)	825 (0.57)	<0.0001[b]		0.7282)
					0.833[c]	0.079	
						(C)	0.9729[c]
							(0.7915-
							1.196)
	Genotype	(n = 599)	(n = 247)	(n = 726)			
	AA	0 (0.00)	0 (0.00)	0 (0.00)		<0.00	
	AG	0 (0.00)	0 (0.00)	0 (0.00)	1.000	01	1.000
rs1534284-	GC	599 (1.00)	247 (1.00)	726 (1.00)	(AV, SV)	(AV,	(0.801-
rs1534283	Allele					SV)	1.235)
(AA/GC;	G	599 (0.50)	247 (0.50)	726 (0.50)	1.000		(AV, SV)
Arg4Gln)	C	599 (0.50)	247 (0.50)	726 (0.50)	(AV, SV)	<0.00	
						01	
						(C)	

'n' represents number of Patients/ Controls,
HWE refers to Hardy-Weinberg Equilibrium,
AV refers to Active Vitiligo, SV refers to Stable Vitiligo and C refers to controls,
[a]Active Vitiligo vs. Stable Vitiligo,
[b]Active Vitiligo vs. Controls,
[c]Stable Vitiligo vs. Controls,
Values are significant at $p \leq 0.025$ due to Bonferroni's correction.

6.3.4 Relative gene expression of *MYG1* in patients with vitiligo and controls

6.3.4 Relative gene expression of *MYG1* in patients with vitiligo and controls

Comparison of the findings showed significant increase in expression of *MYG1* transcripts in 166 vitiligo patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p<0.0001) (Figure 3A). Moreover, generalized vitiligo patients showed significant higher expression of *MYG1* transcripts as compared to localized vitiligo patients (p=0.001) (Figure 3A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.447 fold change in the expression of *MYG1* transcript in patients as compared to controls (Figure 4A).

6.3.5 Correlation of *MYG1* transcripts with the *MYG1* -119C/G genotypes

Further, the expression levels of *MYG1* were analyzed with respect to -119C/G genotypes (Figure 3B). Interestingly, *MYG1* expression was significantly increased in patients with susceptible GG genotypes as compared to controls (p=0.008). Also, patients with genotypes CG showed increased *MYG1* transcripts as compared to controls (p=0.025); however, no significant difference was observed in *MYG1* expression in patients as compared to controls with CC genotypes (p=0.078).

6.3.6 Effect of *MYG1* expression on disease progression

In addition, we also checked the effect of *MYG1* expression on progression of the disease i.e. active and stable cases (Figure 3C). Interestingly, active vitiligo patients showed significant increase in expression of *MYG1* transcripts as compared to the patients with stable vitiligo (p=0.0002) suggesting the involvement of *MYG1* in disease progression. Moreover, the susceptibility of the disease was also checked based on the gender differences and we found that female patients with vitiligo showed significantly higher *MYG1* expression as compared to male patients (p=0.008) (Figure 3D).

When *MYG1*expression was monitored in different age groups of patients, patients with the age group 1-20 yrs showed significantly increased expression of *MYG1* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.037,

p=0.008 and p=0.029 respectively) suggestive of the importance of *MYG*1 in early onset of the disease (Figure 4B).

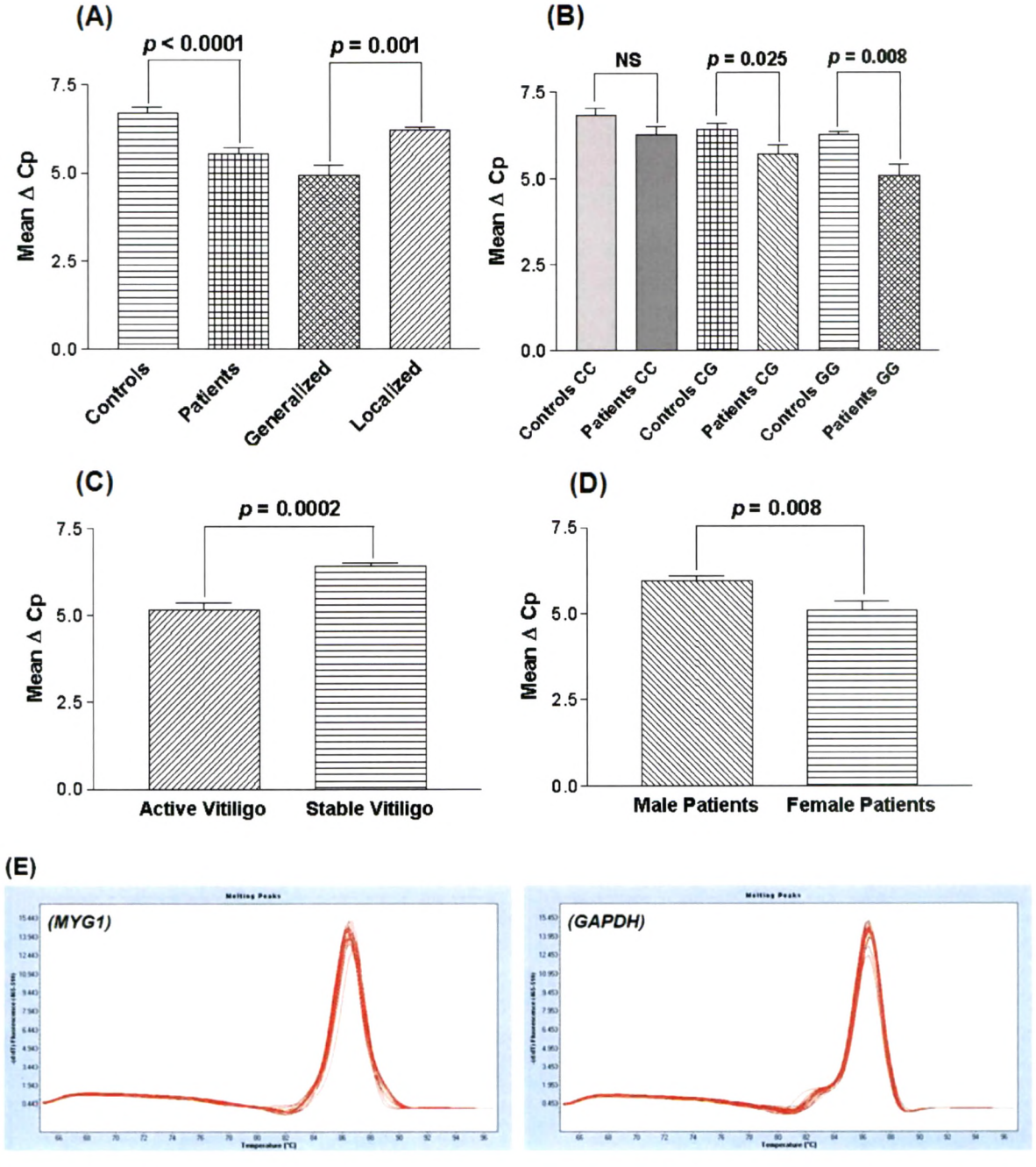

Figure 3. Relative gene expression of *MYG1* in controls and vitiligo patients:

(A) Expression of *MYG1* transcripts in 175 controls, 166 vitiligo patients, 122 generalized vitiligo patients and 44 localized vitiligo patients, as suggested by Mean ΔCp.

(B) Expression of *MYG1* transcripts with respect to *MYG1* promoter -119C/G in 166 vitiligo patients and 175 controls, as suggested by Mean ΔCp.

(C) Expression of *MYG1* transcripts with respect to activity of the disease in 121 patients with active vitiligo and 45 patients with stable vitiligo, as suggested by Mean ΔCp.

(D) Expression of *MYG1* transcripts with respect to gender differences in 87 male patients and 79 female patients with vitiligo, as suggested by Mean ΔCp.

(E) Melt curve analysis of *MYG1* and *GAPDH* showing specific amplification.

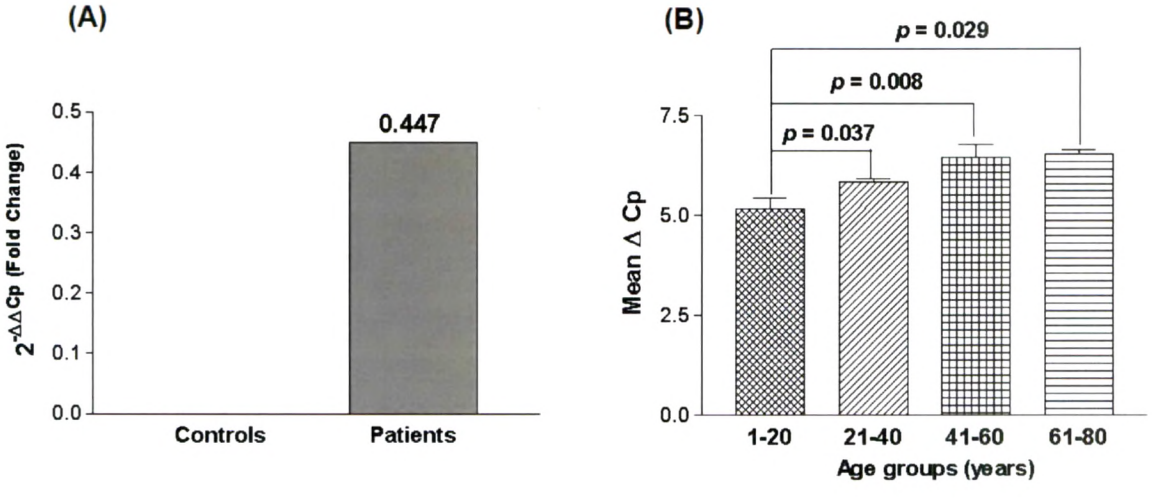

Figure 4. *MYG1* fold change and expression in different age groups of vitiligo:

(A) Expression fold change of *MYG1* transcripts in 166 vitiligo patients against 175 controls showed 0.447 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(B) Expression of *MYG1* transcripts with respect to different age groups in 166 vitiligo patients, as suggested by Mean ΔCp.

6.4 DISCUSSION

Vitiligo cannot be explained by simple Mendelian genetics, however it is characterized by incomplete penetrance, multiple susceptibility loci and genetic heterogeneity (Zhang *et al.*, 2005). It is a complex genetic trait that may include genes involved in melanin biosynthesis, response to oxidative stress and regulation of autoimmunity. The importance of genetic factors for vitiligo susceptibility is evident by reports of its significant familial association (Nordlund, 1997; Kim *et al.*, 1998). Previous study suggests that 22% of Gujarat vitiligo patients exhibit positive family history and 14% patients have at least one first-degree relative affected (Shajil *et al.*, 2006b). Also, in the present study 13.71% of vitiligo patients have one or more first degree relative affected (Table 1) suggesting the involvement of genetic factors in vitiligo pathogenesis.

Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo since it shows frequent association with other autoimmune disorders and patients with the disease show autoantibodies to melanosomal proteins in the serum (Ochi and DeGroot, 1969; Baharav *et al.*, 1996; Kemp *et al.*, 1997). Moreover, autoreactive cytotoxic T lymphocytes (CTLs), which specifically recognize melanocyte differentiation antigens, have been detected in both the peripheral blood and perilesional skin of individuals with vitiligo (Lang *et al.*, 2001; Ogg *et al.*, 1998; Palermo *et al.*, 2001). About 25-30% of generalized vitiligo patients manifest at least one other autoimmune disease; particularly Graves' disease, autoimmune hypothyroidism, rheumatoid arthritis, psoriasis, adult-onset autoimmune diabetes mellitus, pernicious anemia, Addison's disease, and systemic lupus erythematosus (Alkhateeb *et al.*, 2003; Laberge *et al.*, 2005). The destruction of melanocytes due to an autoimmune response in vitiligo can be either through cellular and/or humoral immune response (Shajil *et al.*, 2006a; Kemp *et al.*, 2001). Recently, Singh *et al.* (2012) have shown positive association of HLA-A*33:01, HLA-B*44:03, and HLA-DRB1*07:01 with vitiligo patients from North India and Gujarat suggesting an autoimmune link of vitiligo in these cohorts.

MYG1 gene was initially found to be highly expressed in freely proliferating mouse melanocytes compared with melanoma cells (Smicun, 2000). *MYG1* is predominantly expressed in the ectoderm derived tissues, including epidermis (Philips *et al.*, 2009)

indicating that MYG1 is involved in the development of skin, therefore MYG1 may have crucial role in skin diseases. According to Su *et al.,* (2004) the *MYG1* transcript levels in human skin is under average, compared with other human tissues. However, previous studies indicated that *MYG*1 is upregulated in the skin of patients with vitiligo and atopic eczema (Kingo *et al.*, 2006; Sääf et al., 2008). In the present study, we show that systemic expression of *MYG*1 transcript is elevated in vitiligo patients as compared to controls (Figure 3A). The increased expression of *MYG*1 transcript in generalized vitiligo patients as compared to patients with localized vitiligo (Figure 3A) suggests the crucial role of MYG1 in precipitation of generalized vitiligo. Moreover, the patients with active vitiligo had elevated levels of *MYG*1 transcript as compared to patients with stable vitiligo (Figure 3C) implicating its predominant role in progression of the disease, and our results are in line with the previous findings (Philips *et al.*, 2010; Sääf *et al.*, 2008). In addition, we found elevated *MYG1* transcript levels in the 1-20 yrs of age group of patients (Figure 4B) suggesting its involvement in early phase of the disease.

The gender based analysis had clearly showed the susceptibility of the disease towards female patients as they demonstrated elevated levels of *MYG1* transcript and an early age of onset of vitiligo as compared to male patients (Figure 3D). There are reports suggesting that vitiligo occurs twice as often in females than in males (Westerhof and d'Ischia, 2007). It seems estrogen may be involved in the depigmentation process of vitiligo, as the initiation/progression of the disease is observed at pregnancy, postpartum, in the menopause or after the use of oral contraceptives/hormonal substitution (Schallreuter *et al.*, 2006). Cheng *et al.,* (2006) identified *MYG1* as a putative estrogen receptor alpha target gene; therefore, it is possible that MYG1 can regulate hormonal balance directly through estrogen receptor.

Philips *et al.,* (2010) showed that *MYG1* mRNA levels in skin samples of healthy controls correlate with *MYG1* promoter polymorphism -119C/G, and subjects with homozygous -119G allele have significantly higher *MYG1* transcript levels than subjects with homozygous -119C allele. The study also confirmed higher activity of -119G allele by using *in vitro* luciferase reporter assay and showed alteration of p300 (E1A-associated 300 kDa protein) binding site is a potential reason for the *MYG1*

-119G promoter allele to be more active. The -119G allele was on average more than 2.5 fold more active regardless of the length of the promoter fragment (Philips *et al.*, 2010).

In the present study, we found that -119G allele was more frequent in patients as compared to controls (Table 3) and our results are in line with those of Philips *et. al.* (2010). Further, the genotype-phenotype analysis of -119C/G SNP revealed that patients with GG and CG genotype had increased *MYG1* expression as compared to controls (Figure 3B) suggesting that -119G allele is transcriptionally more active and hence elevated levels of *MYG1* transcripts are seen in patients. Thus, vitiligo patients with -119G promoter allele seem to have genetic susceptibility to vitiligo. However, Philips *et al.*, (2010) showed that the minor allele -119G was related to higher *MYG1* transcript levels only in control group, but not in vitiligo group. Interestingly, in the present study -119G allele was prevalent in active vitiligo patients as compared to patients with stable vitiligo and control group (Table 6) which suggests the crucial role of -119G allele in susceptibility as well as progression of the disease. In addition, we found that patients with *MYG1*-119GG genotype had an early onset of the disease as compared to CG and CC genotypes (Figure 2A) suggesting the participation of MYG1 in early events of the disease. Furthermore, gender based analysis revealed sex-biasness in distribution of -119G allele which is prevalent in female patients as compared to male patients (Table 5) supporting the fact that females are more susceptible for acquring autoimmune diseases like vitiligo.

Genetic association analysis revealed that rs1534284/rs1534283 double-polymorphism was likewise prevalently monogenic in Gujarat population with only *MYG1* 4Arg allele being present (Table 3). Previously, this polymorphism was also found to be monogenic in Estonian population (Philips *et al.*, 2010). According to currently available data, there can be either arginine or glutamine at the fourth position of MYG1 in humans. Philips *et al.*, (2010) showed that only positively charged arginine at the fourth position enables mitochondrial entry of MYG1 which has crucial functions inside mitochondria.

The acidic glutamine disturbs a common property of mitochondrial targeting sequence to form an amphiphilic helical structure that is essential for the effective transport of a mitochondrial protein (Shimoda-Matsubayashi, 1996). According to HapMap database heterozygosity for *MYG1* Arg4Gln polymorphism (rs1534284) has been currently detected only in YRI (Yoruba in Ibadan) population from Nigeria. Among 57 subjects from YRI population who were genotyped for HapMap project, 20 subjects (35.1%) were heterozygous for *MYG1* Arg4Gln polymorphism but there are no subjects who are homozygous for *MYG1* 4Gln allele. According to currently available data, *MYG1* 4Gln allele that disturbs mitochondrial entry of MYG1 has never been detected in the homozygous state. Hence, it was proposed that persons with *MYG1* 4Gln variant do not survive or have a health condition that keeps them out from the study groups (Philips *et al.*, 2010).

The genetic association analysis and expression studies suggest that *MYG1* is one of the genes that in interaction with other genetic and environmental factors is responsible for the development of vitiligo. However, *MYG1* expression in the skin seems to be not specific to melanocytes as *MYG1* expression in cultured melanocytes is lower than expression in full skin biopsy and comparable or equal with *MYG1* expression in cultured fibroblasts derived from the same skin (Philips *et al.*, 2010). Therefore, the precise function of MYG1 in the development of vitiligo is still unclear but the up-regulation of several immune system-related genes after *MYG1* siRNA knockdown in cell culture (Philips *et al.*, 2009) suggests that MYG1 can act as a mediator in the immune processes that are disturbed in vitiligo patients.

Philips *et. al.*, (2009) showed that siRNA mediated knockdown of *MYG1* mRNA resulted in the altered levels of transcripts encoding transcription factors involved in development, tissue patterning and growth as well as immune-related processes. The study (Philips *et al.*, 2009) found that immune reaction-related genes were consistently upregulated after *MYG1* knockdown i.e. *GBP1* (guanylate-binding protein 1), *GBP2* (guanylate binding protein 2), *CD59* (complement regulatory protein) and *UL16*-binding protein 2. Previously, *MYG1* mRNA expression was found to be decreased in $CD8^+$ cytotoxic T-lymphocytes after interleukin-12-induced activation in a mouse mammary carcinoma model (Cao *et al.*, 2004) and in response

to *in vitro* treatment of peripheral blood cells with IFN-α (Taylor *et al.,* 2004). Additionally, upregulation of *MYG1* mRNA has been reported in a methylcholanthrene-induced sarcoma tumour model following treatment with the anti-inflammatory COX (cyclo-oxygenase)-inhibitor indomethacin (Axelsson *et al.,* 2007). These reports support the involvement of MYG1 in immune regulatory functions and hence it becomes pertinent to investigate *MYG1* gene especially in case of generalized vitiligo which is considered to be of autoimmune origin.

In conclusion, our results confirm that -119C/G polymorphism influences *MYG1* transcript levels and minor -119G allele is the risk-allele for the development of vitiligo. The study also emphasizes the influence of MYG1 on the disease progression, onset of the disease and gender biasness for developing vitiligo. More detailed studies will be needed to confirm the genetic association of *MYG1* promoter and structural polymorphisms and to understand the precise function of MYG1 in vitiligo pathogenesis.

6.5 REFERENCES

Alkhateeb A, Fain PR, Thody A, Bennett DC and Spritz RA (2003). Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their relatives. *Pigment Cell Res* 16: 208-214.

Axelsson H, Lo¨ nnroth C, Andersson M, Wang W and Lundholm K (2007). Global tumor RNA expression in early establishment of experimental tumor growth and related angiogenesis following COX-inhibition evaluated by microarray analysis. *Cancer Informatics* 2: 199-213.

Baharav E, Merimski O, Shoenfeld Y, Zigelman R, Gilbrud B, Yecheskel G *et al.* (1996). Tyrosinase as an autoantigen in patients with vitiligo. *Clin Exp Immunol* 105: 84-88.

Cao S, Xiang Z and Ma X (2004). Global gene expression profiling in interleukin-12-induced activation of CD8(+) cytotoxic T lymphocytes against mouse mammary carcinoma. *Cell Mol Immunol* 1: 357-366.

Cheng AS, Jin VX, Fan M, Smith LT, Liyanarachchi S, Yan PS *et al.* (2006). Combinatorial analysis of transcription factor partners reveals recruitment of c-MYC to estrogen receptor-alpha responsive promoters. *Mol Cell* 21: 393-404.

Faul F, Erdfelder E, Lang AG and Buchner A (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* 39: 175-191.

Handa S and Kaur I (1999). Vitiligo: clinical findings in 1436 patients. *J Dermatol*; 10: 653-657.

Hawse JR, Hejtmancik JF, Huang Q, Sheets NL, Hosack DA, Lempicki R *et al.* (2003). Identification and functional clustering of global gene expression differences between human age-related cataract and clear lenses. *Mol Vis* 9: 515-537.

Kemp EH, Gawkrodger DJ, MacNeil S, Watson PF and Weetman AP (1997). Detection of tyrosinase autoantibodies in vitiligo patients using 35S-labelled recombinant human tyrosinase in a radioimmunoassay. *J Invest Dermatol* 109: 69-73.

Kemp EH, Waterman EA, and Weetman AP (2001). Immunological pathomechanisms in vitiligo. *Expert Rev Mol Med* 23: 1.

Kim SM, Chung HS and Hann SK (1998). The genetics of vitiligo in Korean patients. *Int J Dermatol* 37: 908-910.

Kingo K, Philips MA, Aunin E, Luuk H, Karelson M, Rätsep R *et al.* (2006). MYG1, novel melanocyte related gene, has elevated expression in vitiligo. *J Dermatol Sci* 44: 119-122.

Kõks S, Luuk H, Nelovkov A, Areda T and Vasar E (2004). A screen for genes induced in the amygdaloid area during cat odor exposure. *Genes Brain Behav* 3: 80-89.

Laberge G, Mailloux CM, Gowan K, Holland P, Bennett DC, Fain PR *et al.* (2005). Early disease onset and increased risk of other autoimmune diseases in familial generalized vitiligo. *Pigment Cell Res* 18: 300-305.

Lang KS, Caroli CC, Muhm A, Wernet D, Moris A, Schittek B *et al.* (2001). HLA-A2 restricted, melanocyte-specific CD8+ T lymphocytes detected in vitiligo patients are related to disease activity and are predominantly directed against MelanA/MART1. *J Invest Dermatol* 116: 891-897.

Nepomuceno-Silva JL, De Melo LD, Mendonça SM, Paixão JC and Lopes UG (2004). Characterization of Trypanosoma cruzi TcRjl locus and analysis of its transcript. *Parasitology129*: 325-333.

Nordlund JJ (1997). The epidemiology and genetics of vitiligo. Clin Dermatol; 15: 875-878.

Ochi Y and DeGroot LJ. (1969). Vitiligo in Graves' disease. *Ann Intern Med* 71: 935-940.

Ogg GS, Dunbar PR, Romero P, Chen JL and Cerundolo V (1998). High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. *J Exp Med* 188: 1203-1208.

Ortonne JP and Bose SK (1993). Vitiligo: Where do we stand? *Pigment Cell Res* 8: 61-72.

Palermo B, Campanelli R, Garbelli S, Mantovani S, Lantelme E, Brazzelli V *et al.* (2001). Specific cytotoxic T lymphocyte responses against MelanA/MART1, tyrosinase A PTPN22 gene polymorphism in vitiligo and Gp100 in vitiligo by the use of major histocompatibility complex/peptide tetramers: the role of cellular immunity in the etiopathogenesis of vitiligo. *J Invest Dermatol* 117: 326-332.

Philips MA, Kingo K, Karelson M, Rätsep R, Aunin E, Reimann E *et al.* (2010). Promoter polymorphism -119C/G in MYG1 (C12orf10) gene is related to vitiligo susceptibility and Arg4Gln affects mitochondrial entrance of Mygl. *BMC Med Genet* 11: 56.

Philips MA, Vikesaa J, Luuk H, Jonson L, Lilleväli K, Rehfeld JF *et al.* (2009). Characterization of MYG1 gene and protein: subcellular distribution and function. *Biol Cell* 101: 361-373.

Rosenberg SA (1997). Cancer vaccines based on the identification of genes encoding cancer regression antigens. *Immunol Today* 18: 175-182.

Sääf AM, Tengvall-Linder M, Chang HY, Adler AS, Wahlgren CF, Scheynius A *et al.* (2008). Global Expression Profiling in Atopic Eczema Reveals Reciprocal Expression of Inflammatory and Lipid Genes. *PloS One* 3: e4017.

Schallreuter KU, Chiuchiarelli G, Cemeli E, Elwary SM, Gillbro JM, Spencer JD *et al.* (2006). Estrogens can contribute to hydrogen peroxide generation and quinone-mediated DNA damage in peripheral blood lymphocytes from patients with vitiligo. *J Invest Dermatol* 126: 1036-1042.

Shajil EM, Agrawal D, Vagadia K, Marfatia YS and Begum R (2006b). Vitiligo: Clinical profiles in Vadodara, Gujarat. *Ind J Dermatol* 51: 100-104.

Shajil EM, Chatterjee S, Agrawal D, Bagchi T and Begum R (2006a). Vitiligo: Pathomechanisms and genetic polymorphism of susceptible genes. *Ind J Exp Biol* 44: 526-539.

Shimoda-Matsubayashi S, Matsumine H, Kobayashi T, Nakagawa-Hattori Y, Shimizu Y and Mizuno Y (1996). Structural Dimorphism in the Mitochondrial Targeting Sequence in the Human Manganese Superoxide Dismutase Gene A Predictive Evidence for Conformational Change to Influence Mitochondrial Transport and a Study of Allelic Association in Parkinson's Disease. *Biochem Biophys Res Commun* 226: 561-565.

Singh A, Sharma P, Kar HK, Sharma VK, Tembhre MK *et al.* (2012). HLA alleles and amino acid signatures of the peptide binding pockets of HLA molecules in Vitiligo. *J Invest Dermatol* 132: 124-134.

Smicun Y (2000). The Human homologue of *MYG*1 the highly conserved gene from autonomously proliferating mouse melanocytes: *Swiss Institute of Bioinformatics.*

Spritz RA (2011). Recent progress in the genetics of generalized vitiligo. *J Genet and Genom* 38: 271-278.

Spritz RA (2007). The genetics of generalized vitiligo and associated autoimmune diseases. *Pigment Cell Res* 20: 271-278.

Spritz RA (2008). The genetics of generalized vitiligo. *Curr Dir Autoimmunity* 10: 244-257.

Spritz RA (2010). The genetics of generalized vitiligo: autoimmune pathways and an inverse relationship with malignant melanoma. *Genome Medicine* **2**: 78.

Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D *et al.* (2004). A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci U S A* 101: 6062-6067.

Taieb A, Picardo M and VETF Members (2007). The definition and assessment of vitiligo: a consensus report of the Vitiligo European Task Force. *Pigment Cell Res* 20: 27-35.

Taylor MW, Grosse WM, Schaley JE, Sanda C, Wu X, Chien SC *et al.* (2004). Global effect of PEG-IFN-α and ribavirin on gene expression in PBMC invitro. *J Interferon Cytokine Res* 24: 107-118.

Westerhof W and d'Ischia M (2007). Vitiligo puzzle: the pieces fall in place. *Pigment Cell Res.* 20: 345-359.

Zhang XJ, Chen JJ and Liu JB (2005). The genetic concept of vitiligo. *J Dermatol Sci* 39: 137-146.