CHAPTER VII

NACHT LEUCINE-RICH REPEAT PROTEIN 1 (*NALP1*) GENE PROMOTER AND STRUCTURAL POLYMORPHISMS AND THEIR GENOTYPE-PHENOTYPE CORRELATION WITH GENERALIZED VITILIGO SUSCEPTIBILITY

7.1 INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder in which progressive, patchy loss of pigmentation from skin, overlying hair, and oral mucosa results from autoimmune loss of melanocytes from the involved areas (Nordlund *et al.*, 2006). It appears to be a polygenic, multifactorial disorder involving both multiple susceptibility genes (Majumder *et al.*, 1993; Nath *et al.*, 1994; Sun *et al.*, 2006), perhaps in different combinations in different populations, and as-yet unknown environmental triggers. Epidemiological studies have shown frequent family clustering of vitiligo cases, with elevated risk of vitiligo in first-degree relatives and high concordance in monozygotic twins (Alkhateeb *et al.*, 2003; Sun *et al.*, 2006). Approximately one in four vitiligo patients manifest another autoimmune disorder, principally autoimmune thyroid disease, 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). Furthermore, these autoimmune diseases also occur at elevated frequency among vitiligo patient's close relatives, suggesting that increased risk of vitiligo and other autoimmune disorders in these families has a genetic basis.

A number of genes have been implicated in the pathogenesis of generalized vitiligo on the basis of genetic linkage and association studies (reviewed in Spritz, 2008). Recent genome-wide association studies (GWAS) revealed a highly significant association of familial cases of generalized vitiligo with polymorphic variants of the gene encoding *NALP1* - a key regulator of the innate immune system (also known as *NLRP1*; Ting *et al.*, 2008) (Jin *et al.*, 2007a).

NALP1 is a critical component of the surveillance system that detects bacterial and viral infections thus acts as a primary regulator of the innate immune system. *NALP1* is mainly expressed in Langerhans cells and T cells (Kummer *et al.*, 2007). NALP1 is thought to recognize pathogen-associated molecular patterns, which would trigger the assembly of the NALP1 inflammosome and consequently stimulate inflammatory response and apoptotic pathways (Church *et al.*, 2008).

The *NALP1* gene encodes NLRP1, a leucine-rich repeat protein 1, a member of the nucleotide oligomerization domains-like receptors (NLRs) family. The NLRs are a group of cytoplasmic pattern recognition receptors, which nonspecifically recognize microbial products, such as lipopolysaccharide, thereby stimulating innate immunity. NALP1 contains five domains, a protein-protein interaction N-terminal pyrin domain followed by centrally located NACHT (NAIP CIIA HET-E and TP1 family proteins) domain responsible for oligomerization and activation of the NLRs, a domain comprised of five tandem leucine-rich repeat (LRR) domains that is speculated to bind microbial ligands, a FIIND domain, and a finally a C-terminal caspase recruitment (CARD) domain (Martinon and Tschopp, 2005). Activated NALP1 interacts directly with caspase-1 and caspase-5, forming a multiprotein complex known as the inflammasome, which promotes the processing and maturation of cytokines such as IL1, IL18, and IL33 (Tschopp *et al.*, 2003; Faustin *et al.*, 2007). Through its multiple domain structure, NALP1 plays a crucial role in the assembly of the apoptosome, a complex composed of NALP1, caspase-2, and caspase-9. High levels of expression in immune cells, particularly T cells and Langerhans' cells, emphasize the role of NLRP1 in regulation of the immune system (Ting and Davis, 2005).

Earlier studies showed variants of *NALP1* to be associated with vitiligo in Caucasian patients from the USA, UK, Romania and Jordanian populations (Jin *et al.*, 2007a,b,; Alkhateeb and Qarqaz, 2010). Other studies have also demonstrated that polymorphisms in *NLRP*1 are associated with autoimmune conditions like Addison's disease, and type 1 diabetes (Magitta *et al.*, 2009; Zurawek *et al.*, 2010).

The objectives of this study were:

i.) To measure and compare *NALP1* transcript levels in patients with vitiligo and in unaffected controls.

ii.) To determine whether *NALP1* promoter (A/G; rs2670660), structural (A/T; rs12150220) and intronic (T/C rs6502867) polymorphisms are associated with vitiligo susceptibility and modulate *NALP1* transcript levels in these groups.

iii.) To correlate *NALP1* polymorphisms/levels with the onset and progression of disease.

7.2 MATERIALS AND METHODS

7.2.1 Study Subjects:

The study group included 537 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 237 males and 300 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 diseases. A total of 645 ethnically sex-matched unaffected individuals (295 males and 350 females) were included 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 Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

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

	Generalized Vitiligo (GV) Patients	**Controls**
	(n = 537)	(n = 645)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	237 (44.13%)	295 (45.74%)
Female	300 (55.87%)	350 (54.26%)
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		
Active vitiligo	392 (73.00%)	NA
Stable vitiligo	145 (27.00%)	NA
Family history	71 (13.22%)	NA

7.2.2 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.

7.2.3 Genotyping of *NALP1* A/G promoter (rs2670660) and T/C intronic (rs6502867) polymorphisms:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype A/G (rs2670660) and T/C (rs6502867) polymorphisms of *NALP1* gene (Figure 1A & B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

Restriction enzymes (New England Biolabs, Beverly, MA) *Apo*I and *Hpy*8I were used for digesting amplicons of A/G (rs2670660) and T/C (rs6502867) of *NALP1* gene respectively (Table 2). 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 50 bp or 100 bp 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 *NALP1* A/G promoter (rs2670660) and T/C intronic (rs6502867) SNPs genotyping and gene expression analyses.

Gene/SNP Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs2670660)				*Apo*I
NALP1 A/G F	TGTTTATCCCAGGGCTTCTTGT	53	227	(179 &
NALP1 A/G R	CAGTGTTCTGTGGAAATGAAAGAG			48 bp)
(rs6502867)				*Hpy*8I
NALP1 T/C F	TGGGGGTTTGGGTTCTTAG	56	216	(125 &
NALP1 T/C R	TTTCTCATGATAGTTTGGGTGTG			91 bp)
NALP1 expression F	CGCCGCTGGAGAGAAATCTC	65	232	-
NALP1 expression R	CTCTCTCTTTCTCTGATTTCTGTGT			
GAPDH expression F	ATCCCATCACCATCTTCCAGGA	65	122	-
GAPDH expression R	CAAATGAGCCCCAGCCTTCT			

7.2.4 Genotyping of *NALP1* A/T structural (rs12150220; Leu155His) polymorphism:

The genotyping of A/T (rs12150220) SNP of *NALP1* was carried out by dual color hydrolysis probes (FAM and VIC) using LightCycler® 480 Real-Time PCR protocol with background corrected end point fluoroscence analysis using TaqMan SNP genotyping assay (Assay ID: C_1600653_10; Life Technologies Corp., California, USA). Real-time PCR was performed in 10 µl volume using LightCycler® 480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany) following the

manufacturer's instructions. A no-template control (NTC) was used with the SNP genotyping assay.

7.2.5 Determination of *NALP1* and *GAPDH* mRNA expression:

7.2.5.1 RNA extraction and cDNA synthesis:

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- 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).

7.2.5.2 Real-time PCR:

The expression of *NALP1* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (94°C for 15 s, 65°C for 20 s, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed (Figure 4I). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

7.2.6 Statistical analyses:

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all three polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis. The distribution of the genotypes and allele frequencies of *NALP1* polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.017 were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated.
Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi *et al.*, 2005). The linkage disequilibrium (LD) coefficients D' = D/Dmax and r^2-values for the pair of the most common alleles at each site were estimated using the Haploview programe version 4.1 (Barrett *et al.*, 2005). Age of onset analysis and relative expression of *NALP1* 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).

7.3 RESULTS

7.3.1 Analysis of association between *NALP1* (A/G; rs2670660) promoter polymorphism and susceptibility to vitiligo:

PCR-RFLP for *NALP1* A/G promoter polymorphism yielded a 227 bp undigested product corresponding to 'G' allele and 179 bp and 48 bp digested products corresponding to 'A' allele. The three genotypes identified by 2.5% agarose gel electrophoresis were: AA homozygous, AG heterozygous and GG homozygous for A/G polymorphism of *NALP1* gene (Figure 1A).

The genotype and allele frequencies of the A/G polymorphism in 537 vitiligo patients and 645 controls are summarized in Table 3. The *NALP1* A/G promoter

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). In particular, the minor allele (G) of the *NALP1* A/G polymorphism was more frequent in the vitiligo group compared to the control group (51.0% versus 42.0%, p<0.0001) (Table 3). Both control and patient groups were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.062 and p=0.135 respectively) (Table 3).

Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the minor 'G' allele of *NALP1* A/G promoter polymorphism occurred prevalently in the group of patients with active vitiligo (56.0% versus 42.0%, p<0.0001) compared to the control group (Table 4). However, there was no statistically significant difference in the distribution of the 'G' allele between patients with stable vitiligo and control group (p=0.235) (Table 4). Interestingly, the 'G' allele was more prevalent in patients with active vitiligo as compared to stable vitiligo (56.0% versus 38.0%, p<0.0001) suggesting the important role of 'G' allele in progression of the disease. In addition, gender based analysis of *NALP1* A/G polymorphism suggested significant association of minor 'G' allele, consistent with a susceptibility effect with female vitligo patients as compared to male patients (55.0% versus 47.0%, p=0.007, OR: 1.399, 95% CI: 1.099-1.782 (Table 5). This study has 86.71% 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.

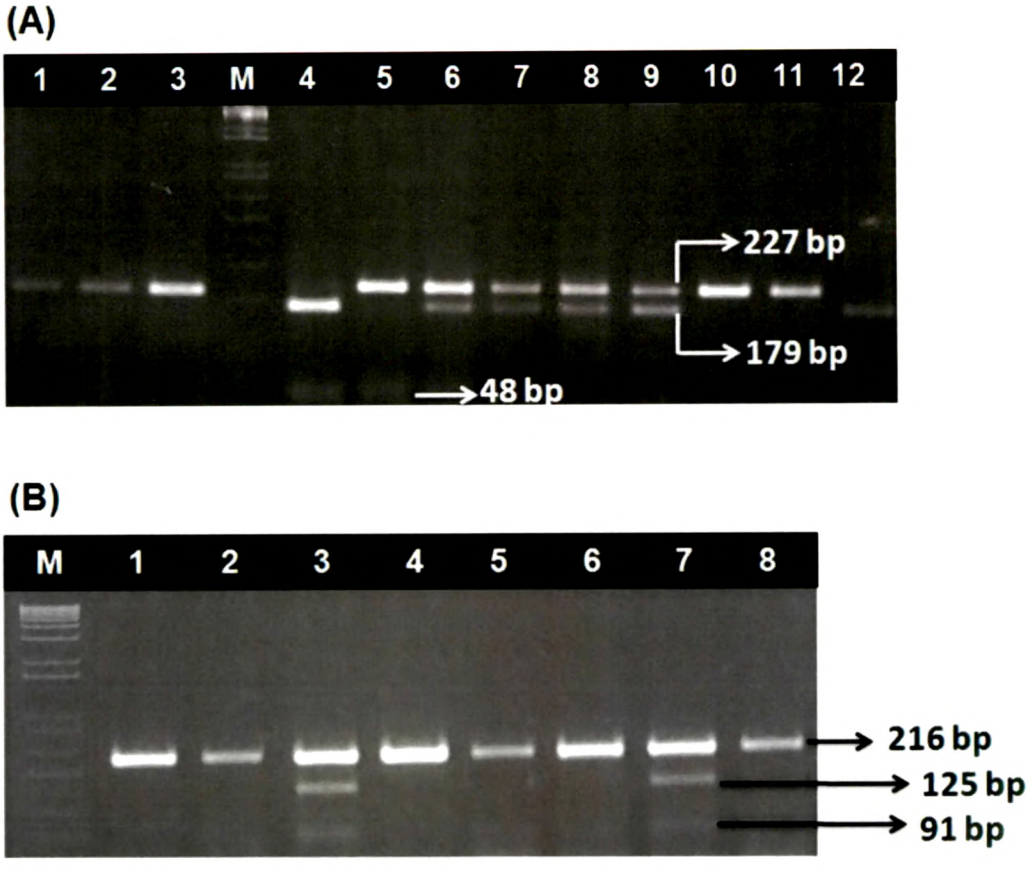


Figure 1. PCR-RFLP analysis of *NALP1* A/G (rs2670660) and C/T (rs6502867) polymorphisms:

(A) PCR-RFLP analysis of *NALP1* A/G (rs2670660) promoter polymorphism on 2.5 % agarose gel electrophoresis: lanes: 4 &12 show homozygous (AA) genotypes; lanes: 6, 7, 8, & 9 show heterozygous (AG) genotypes; lanes: 1, 2, 3, 5, 10 & 11 show homozygous (GG) genotypes; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *NALP1* C/T (rs6502867) intron polymorphism on 2.5 % agarose gel electrophoresis: lanes: 1, 2, 4, 5, 6 & 8 show homozygous (CC) genotypes; lanes: 3, & 7 show heterozygous (CT) genotypes; lane M shows 50 bp DNA ladder.

7.3.2 Analysis of association between *NALP1* (T/C; rs6502867) polymorphism and susceptibility to vitiligo:

PCR-RFLP for *NALP1* T/C intron polymorphism yielded a 216 bp undigested product corresponding to 'C' allele and 125 bp and 91 bp digested products corresponding to 'T' allele. The three genotypes identified by 2.5% agarose gel electrophoresis were: TT homozygous, TC heterozygous and CC homozygous for T/C polymorphism of *NALP1* gene (Figure 1B).

The genotype and allele frequencies of the T/C polymorphism in 537 vitiligo patients and 645 controls are summarized in Table 3. The *NALP1* T/C 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). In particular, the minor allele 'C' of the *NALP1* T/C polymorphism was more frequent in the vitiligo group compared to the control group (41.0% versus 31.0%, $p<0.0001$) (Table 3). Both control and patient groups were found to be in Hardy-Weinberg equilibrium for this polymorphism ($p=0.096$ and $p=0.071$ respectively) (Table 3).
Analysis based on the stage of progression of vitiligo revealed that the increased frequency of the minor 'C' allele of *NALP1* T/C polymorphism occurred prevalently in the group of patients with active vitiligo (44.0% versus 31.0%, $p<0.0001$) compared to the control group (Table 4). However, there was no statistically significant difference in the distribution of the 'C' allele between patients with stable vitiligo and control group (p=0.676) (Table 4). Interestingly, the 'C' allele was more prevalent in patients with active vitiligo as compared to stable vitiligo (44.0% versus 33.0%, p=0.001) suggesting the important role of 'C' allele in progression of the disease. In addition, gender based analysis of *NALP1* T/C polymorphism suggested significant association of minor 'C' allele, consistent with a susceptibility effect with female vitligo patients as compared to male patients (45.0% versus 35.0%, p=0.001, OR 1.553, 95% CI 1.212-1.990 (Table 5). This study has 86.71% 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.

7.3.3 Analysis of association between *NALP1* (A/T; rs12150220) polymorphism and susceptibility to vitiligo:

Real time PCR based TaqMan SNP genotyping method was carried out by dual color hydrolysis probes labbled with FAM (for 'T' allele) and VIC (for 'A' allele) fluorophores for *NALP1* A/T (leu155his) polymorphism which yielded the three genotypes (AA homozygous, AT heterozygous and TT homozygous) as identified by scattered plot using background corrected end point fluoroscence analysis (Figure 2). The genotype and allele frequencies of the *NALP1* A/T (rs12150220) polymorphism in 537 vitiligo patients and 645 controls are summarized in Table 3. The *NALP1* A/T polymorphism was not found to be in significant association with vitiligo patients as suggested by the genotype and allele frequencies for the polymorphism (p=0.074; p=0.012) (Table 3). Both control and patient populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.052 and p=0.728 respectively) (Table 3).

Analysis based on the stage of progression of vitiligo revealed that there was no significant difference in the distribution of minor 'T' allele of *NALP1* A/T polymorphism between patients with active vitiligo and control group (p=0.0.063) (Table 4). Also, there was no statistically significant difference in the distribution of the 'T' allele between both the groups of patients with active and stable vitiligo and control group (p=1.00; p=0.215) (Table 4). No significant difference was obsereved in 'T' allele frequency between male and female patients (p=0.527) (Table 5).This study has 86.71% statistical power for the effect size 0.08 to detect the association of *NALP1* A/T (leu155his) polymorphism at $p<0.05$ in generalized vitiligo patients and control population.

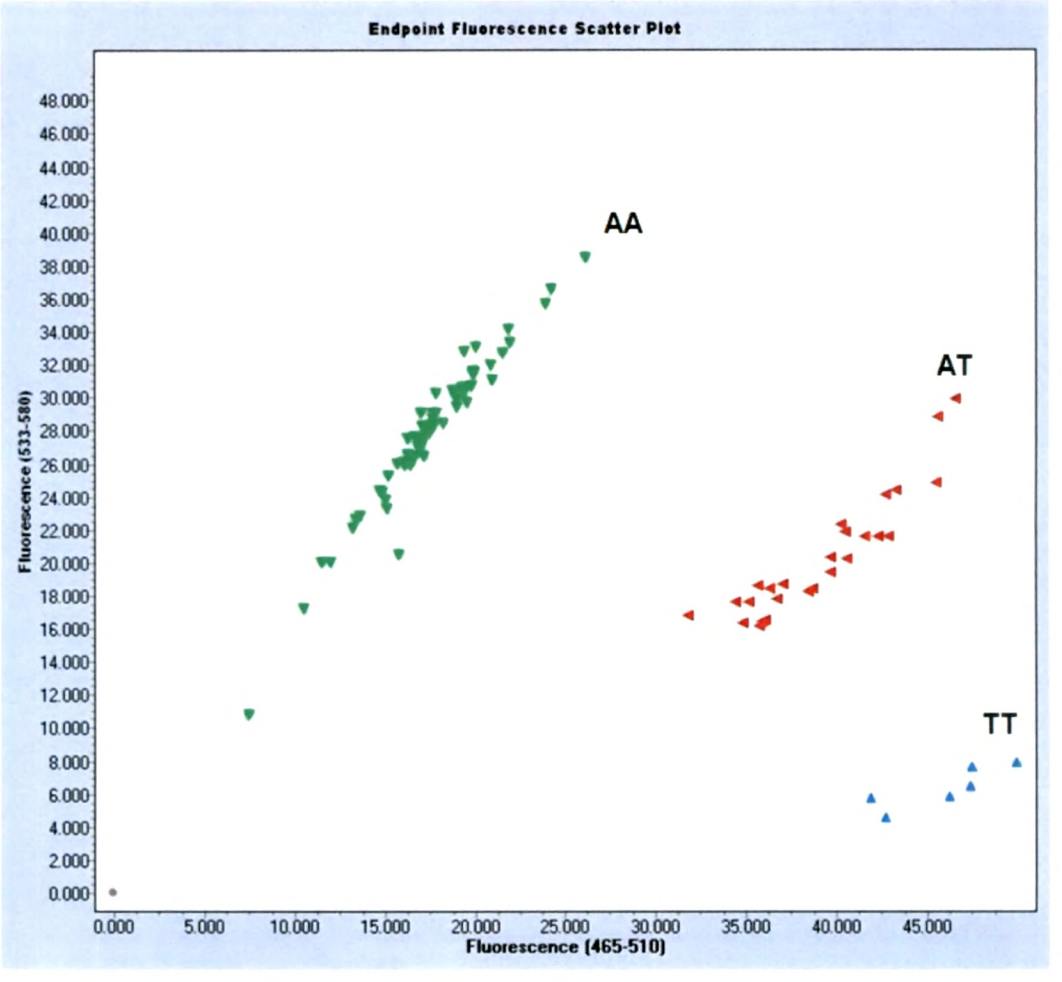


Figure 2. TaqMan end point fluoroscence analysis for *NALP1* A/T (rs12150220) using dual color hydrolysis probes (FAM and VIC) by LightCycler® 480Real-Time PCR protocol. The three genotypes identified as: AA, AT and TT, based on fluorescence with Channel 465-510 (FAM for 'T' allele) and Channel 536-580 (VIC for 'A' allele). A no-template control (NTC) was used with the SNP genotyping assay.

Table 3. Association studies for *NALP1* gene, A/G (rs2670660) promoter, T/C (rs6502867) intron and A/T (rs12150220) 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 = 537)	(n = 645)			
	AA	136 (0.25)	229 (0.36)		0.135	
rs2670660	AG	251 (0.47)	291 (0.45)	<0.0001[a]	(P)	
(A/G)	GG	150 (0.28)	125 (0.19)		0.062	
	Allele				(C)	0.6856
	A	523 (0.49)	749 (0.58)	<0.0001[b]		(0.5825-
	G	551 (0.51)	541 (0.42)			0.8069)
	Genotype	(n = 537)	(n = 645)			
	TT	199 (0.37)	312 (0.48)		0.071	
rs6502867	TC	239 (0.44)	260 (0.40)	<0.0001[a]	(P)	
(T/C)	CC	99 (0.18)	73 (0.12)		0.096	
	Allele				(C)	0.6695
	T	637 (0.59)	884 (0.69)	<0.0001[b]		(0.5652-
	C	437 (0.41)	406 (0.31)			0.7929)
	Genotype	(n= 537)	(n = 645)			
	AA	298 (0.55)	400(0.62)		0.728	
rs12150220	AT	202 (0.38)	205 (0.32)	0.074[a]	(P)	
(A/T)	TT	37 (0.07)	40 (0.06)		0.052	
	Allele				(C)	0.7813
	A	788 (0.73)	1005(0.78)	0.012[b]		(0.6469-
	T	286 (0.27)	285 (0.22)			0.9436)

'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 $p \leq 0.017$ due to Bonferroni's correction.

Table 4. Association studies for *NALP1* gene, A/G (rs2670660) promoter, T/C (rs6502867) intron and A/T (rs12150220) 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 = 392)	(n = 145)	(n = 645)			0.4753 [a]
	AA	77 (0.20)	59 (0.40)	229(0.36)	<0.0001[a]	0.686	(0.3606-
rs2670660	AG	189 (0.48)	62 (0.43)	291(0.45)	<0.0001[b]	(AV)	0.6264)
(A/G)	GG	126 (0.32)	24 (0.17)	125(0.19)	0.467[c]		0.5618 [b]
						0.268	(0.4696-
	Allele					(SV)	0.6721)
	A	343 (0.44)	180 (0.62)	749(0.58)	<0.0001 [a]		1.182[c]
	G	441 (0.56)	110 (0.38)	541(0.42)	<0.0001[b]	0.062	(0.9097-
					0.235[c]	(C)	1.536)
	Genotype	(n = 392)	(n = 145)	(n = 645)			0.6296 [a]
	TT	129 (0.33)	70 (0.48)	312(0.48)	0.004[a]	0.366	(0.4744-
	TC	184 (0.47)	55 (0.38)	260(0.40)	<0.0001[b]	(AV)	0.8356)
rs6502867	CC	79 (0.20)	20 (0.14)	73 (0.12)	0.676[c]		1.685 [b]
	Allele					0.094	(1.402-
(T/C)	T	442 (0.56)	195 (0.67)	884(0.69)	0.001 [a]	(SV)	2.024)
	C	342 (0.44)	95 (0.33)	406(0.31)	<0.0001[b]		1.061 [c]
					0.676[c]	0.096	(0.8081-
						(C)	1.392)
	Genotype	(n = 392)	(n = 145)	(n = 645)			0.9871 [a]
	AA	216 (0.55)	82 (0.57)	400 (0.62)	0.843[a]	0.995	(0.7248-
rs12150220	AT	150 (0.38)	52 (0.36)	205 (0.32)	0.081[b]	(AV)	1.344)
(A/T)	TT	26 (0.07)	11 (0.07)	40 (0.06)	0.464[c]		0.8171 [b]
	Allele					0.496	(0.6641-
	A	582 (0.74)	216 (0.74)	1005(0.78)	1.000 [a]	(SV)	1.005)
	T	202 (0.26)	74 (0.26)	285 (0.22)	0.063[b]		1.208 [c]
					0.215[c]	0.052	(0.8994-
						(C)	1.623)

'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 $p \leq 0.017$ due to Bonferroni's correction.

Table 5. Association studies for *NALP1* gene, A/G (rs2670660) promoter, T/C (rs6502867) intron and A/T (rs12150220) 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 = 237)	(n = 300)			
	AA	74 (0.31)	62 (0.20)	0.017[a]	0.091	
rs2670660	AG	105 (0.44)	146 (0.50)		(M)	
(A/G)	GG	58 (0.25)	92 (0.30)			
	Allele				0.771	
	A	253 (0.53)	270 (0.45)	0.007[b]	(F)	1.399
	G	221 (0.47)	330 (0.55)			(1.099-1.782)
	Genotype	(n = 237)	(n = 300)			
	TT	105 (0.44)	94 (0.31)		0.220	
rs6502867	TC	99 (0.42)	140 (0.47)	0.003[a]	(M)	
(T/C)	CC	33 (0.14)	66 (0.22)			
	Allele				0.311	
	T	309 (0.65)	328 (0.55)	0.001[b]	(F)	1.553
	C	165 (0.35)	272 (0.45)			(1.212-1.990)
	Genotype	(n= 237)	(n = 300)			
	AA	136 (0.57)	162 (0.54)		0.586	
rs12150220	AT	85 (0.36)	117 (0.39)	0.729[a]	(M)	
(A/T; Leu155His)	TT	16 (0.07)	21 (0.07)			
	Allele				0.984	
	A	357 (0.75)	441 (0.74)	0.527[b]	(F)	1.100
	T	117 (0.25)	159 (0.26)			(0.8343-1.451)

'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 $p \leq 0.017$ due to Bonferroni's correction.

7.3.4 Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the three polymorphisms investigated in the *NALP1* gene were in low LD association [**A/G: T/C** (D' =0.082, r^2=0.003); **A/G: A/T** (D'= 0.206, r^2=0.020); **T/C: A/T** (D'= 0.089, r^2=0.006)]. A haplotype evaluation of the three polymorphic sites was performed and the estimated frequencies of the haplotypes differed significantly between vitiligo patients and controls (global *p*-value=1.54e-008) (Table 6).

Table 6. Distribution of haplotypes frequencies for *NALP1* gene, A/G (rs2670660) promoter, T/C (rs6502867) intron and A/T (rs12150220; leu155his) polymorphisms among generalized vitiligo patients and controls.

Haplotype (A/G (rs2670660), T/C (rs6502867) and A/T (rs12150220)	Generalized Vitiligo Patients (Freq. %) (n=966)	Controls (Freq. %) (n=1066)	*p* for Association	$p_{(global)}$	Odds ratio (95% CI)
A C A	142.63(0.148)	170.64(0.160)	0.444	1.54e-008	0.910 [0.715~1.159]
A C T	41.91(0.043)	56.25(0.053)	0.327		0.815 [0.541~1.228]
A T A	196.76(0.204)	312.78(0.293)	3.42e-006		0.617 [0.503~0.757]
A T T	73.70(0.076)	85.33(0.080)	0.759		0.950 [0.687~1.315]
G C A	100.25(0.104)	69.26(0.065)	0.002		1.668 [1.212~2.296]
G C T	92.22(0.095)	51.85(0.049)	3.94e-005		2.067 [1.453~2.939]
G T A	205.37(0.213)	235.32(0.221)	0.665		0.954 [0.772~1.179]
G T T	112.17(0.116)	84.57(0.079)	0.01		1.526 [1.134~2.054]

CI represents Confidence Interval,

(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

Interestingly, the frequency of susceptible haplotype 'GCT' containing the minor alleles of all the three polymorphisms were significantly higher in vitiligo patients as compared to controls (9.5% vs 4.9%; p=3.94e-005) and increased the risk of vitiligo by 2.0-fold [odds ratio (OR): 2.067; 95% confidence interval (CI): (1.453~2.939)] (Table 6). Also two other haplotypes: 'GCA' and 'GTT' were significantly increased in vitiligo patients as compared to controls (p=0.002, p=0.01). However, the wild type haplotype 'ATA' was more frequently observed in control group as compared to the patient group (20% vs 29.3%; p= 3.42e-006) (Table 6).

7.3.5 Effect of *NALP1* polymorphisms on age of onset of vitiligo:

When age of onset of the disease was correlated with the *NALP1* A/G (rs2670660) promoter genotypes, patients with susceptible GG genotypes showed early onset of the disease as compared to AA and AG genotypes (p<0.0001 and p=0.011 respectively) (Figure 3A). Moreover, patients with genotype AG showed early onset of the disease as compared to AA genotypes (p=0.003) (Figure 3A) suggesting the effect of the susceptible allele 'G' on the early onset of disease.

Analysis of age of onset with *NALP1* T/C (rs6502867) genotypes revealed that patients with susceptible CC genotypes showed early onset of the disease as compared to TT and TC genotypes (p<0.0001 and p=0.0003 respectively) (Figure 3B). Moreover, patients with genotype TC showed early onset of the disease as compared to TT genotypes (p=0.032) (Figure 3B) suggesting the effect of the susceptible allele 'C' on the early onset of disease.

However, patients with *NALP1* (rs12150220) AA genotypes did not show any significant difference for the age of onset of the disease as compared to TT (p=0.163) and AT genotypes (p=0.635) (Figure 3C). Also, patients with AT genotype did not show significant difference in age of onset of disease as compared to TT genotype (p=0.357) (Figure 3C).

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<0.0001) (Figure 3D).

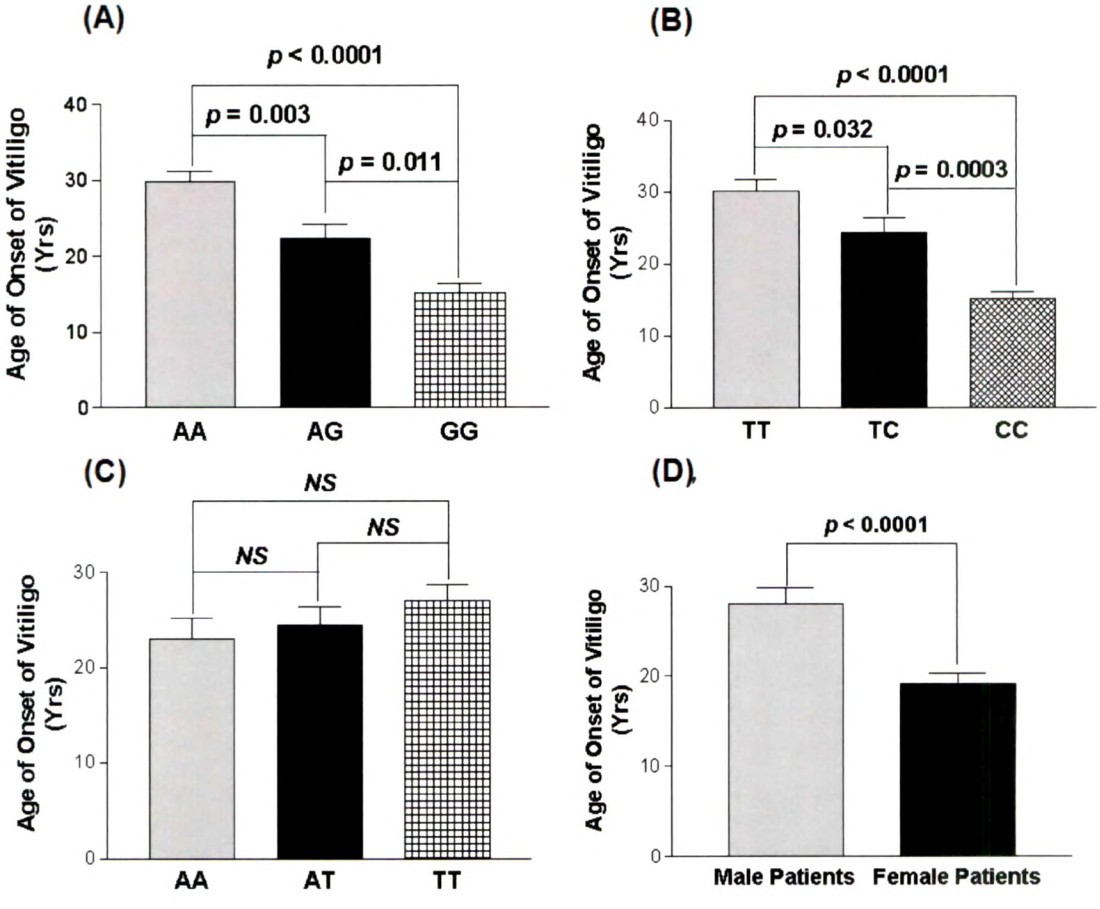


Figure 3. Age of onset of the disease in vitiligo patients:

(A) Comparison of age of onset of the disease (Years) with respect to *NALP1* A/G (rs2670660) polymorphism in 537 vitiligo patients. Vitiligo patients with GG genotype showed early age of onset of disease as compared to AA (Mean age of onset ± SEM: 15.13 ± 1.342 vs 29.77 ± 1.497; p<0.0001) and AG genotypes (Mean age of onset ± SEM: 15.13 ± 1.342 vs 22.42 ± 1.767; p=0.011). Patients with AG genotype showed early age of onset of disease as compared to AA genotype (Mean age of onset ± SEM: 22.42 ± 1.767 vs 29.77 ± 1.497; p=0.003).

(B) Comparison of age of onset of the disease (Years) with respect to *NALP1* T/C (rs6502867) polymorphism in 537 vitiligo patients. Vitiligo patients with CC genotype showed early age of onset of disease as compared to TT (Mean age of onset ± SEM: 15.13 ± 1.045 vs 30.13 ± 1.544; p<0.0001) and TC genotypes (Mean age of onset ± SEM: 15.13 ± 1.045 vs 24.40 ± 2.032; p=0.0003). Patients with TC genotype

showed early age of onset of disease as compared to TT genotype (Mean age of onset ± SEM: 24.40 ± 2.032 vs 30.13 ± 1.544; p=0.032).

(C) Comparison of age of onset of the disease (Years) with respect to *NALP1* A/T (rs12150220) polymorphism in 537 vitiligo patients. No significant diiference was observed in age of onset between Vitiligo patients with AA and TT genotypes (Mean age of onset ± SEM: 23.09 ± 2.097 vs 27.02 ± 1.874; p=0.163) and AT genotypes (Mean age of onset ± SEM: 23.09 ± 2.097 vs 24.48 ± 2.000; p=0.635). Also, patients with AT genotype did not show significant difference in age of onset of disease as compared to TT genotype (Mean age of onset ± SEM: 24.48 ± 2.000 vs 27.02 ± 1.874; p=0.357).

(D) Comparison of age of onset of the disease (Years) with respect to gender differences in 237 male patients and 300 female patients with vitiligo. Female patients showed an early age of onset of disease as compared to male patients (Mean age of onset ± SEM: 19.20 ± 1.091 vs 28.08 ± 1.796; p<0.0001).

7.3.6 Relative gene expression of *NALP1* in patients with vitiligo and controls:

Comparison of the findings showed significant increase in expression of *NALP1* transcripts in 122 vitiligo patients than in 175 unaffected controls after normalization with *GAPDH* expression as suggested by mean ΔCp values (p<0.0001) (Figure 4A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.132 fold change in the expression of *NALP1* transcript in patients as compared to controls (Figure 4B).

7.3.6.1 Correlation of *NALP1* transcripts with the investigated polymorphisms:

Further, the expression levels of *NALP1* were analyzed with respect to A/G (rs2670660) promoter genotypes (Figure 4C). Interestingly, *NALP1* expression was significantly increased in patients with susceptible GG genotypes as compared to controls (p=0.001). Also, patients with genotypes AG showed increased *NALP1* transcripts as compared to controls (p=0.007); however, no significant difference was observed in *NALP1* expression in patients as compared to controls with AA genotypes (p=0.102).

The genotype-phenotype correlation for T/C (rs6502867) showed significant increased expression of *NALP*1 with CC and TC genotypes in patients as compared to those of controls (p=0.020; p=0.006); however, no significant difference was observed in *NALP1* expression in patients as compared to controls with TT genotypes (*p*=0.121) (Figure 4D). The genotype-phenotype analysis for A/T (rs12150220) SNP did not show any significant difference in *NALP*1 expression between patients and controls (p=0.082; p=0.162; p=0.160) (Figure 4E).

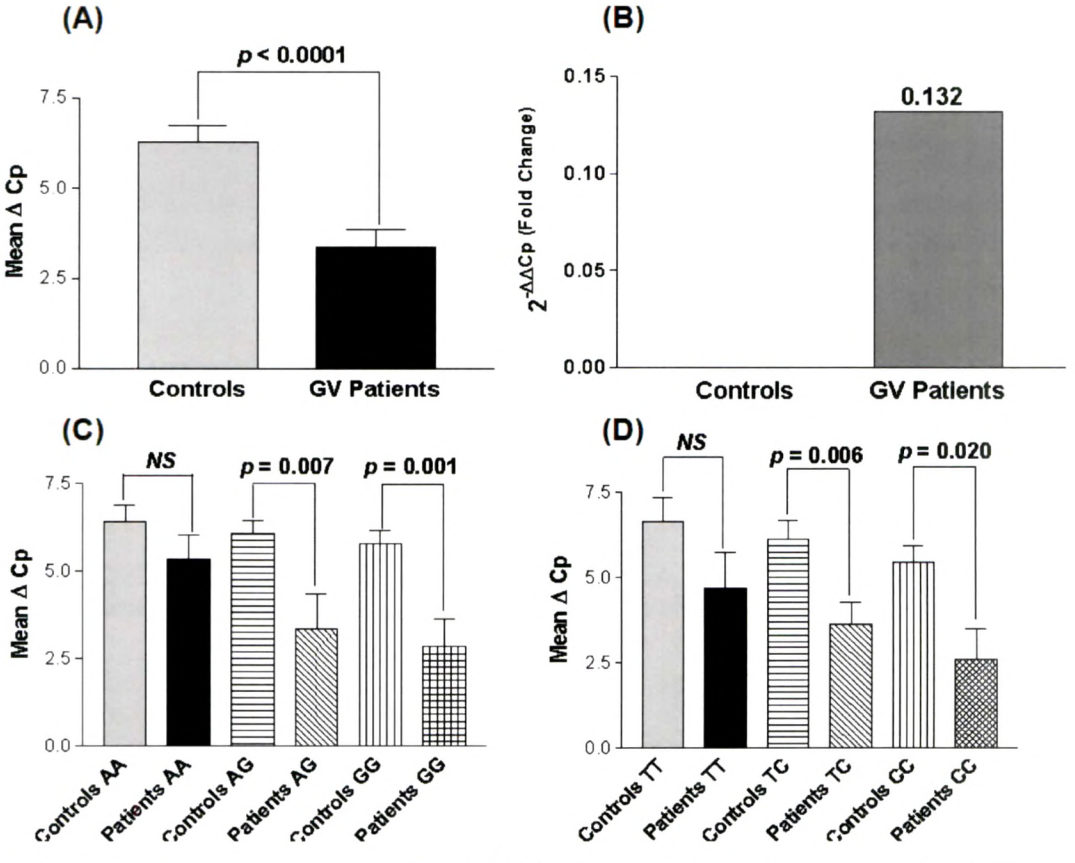


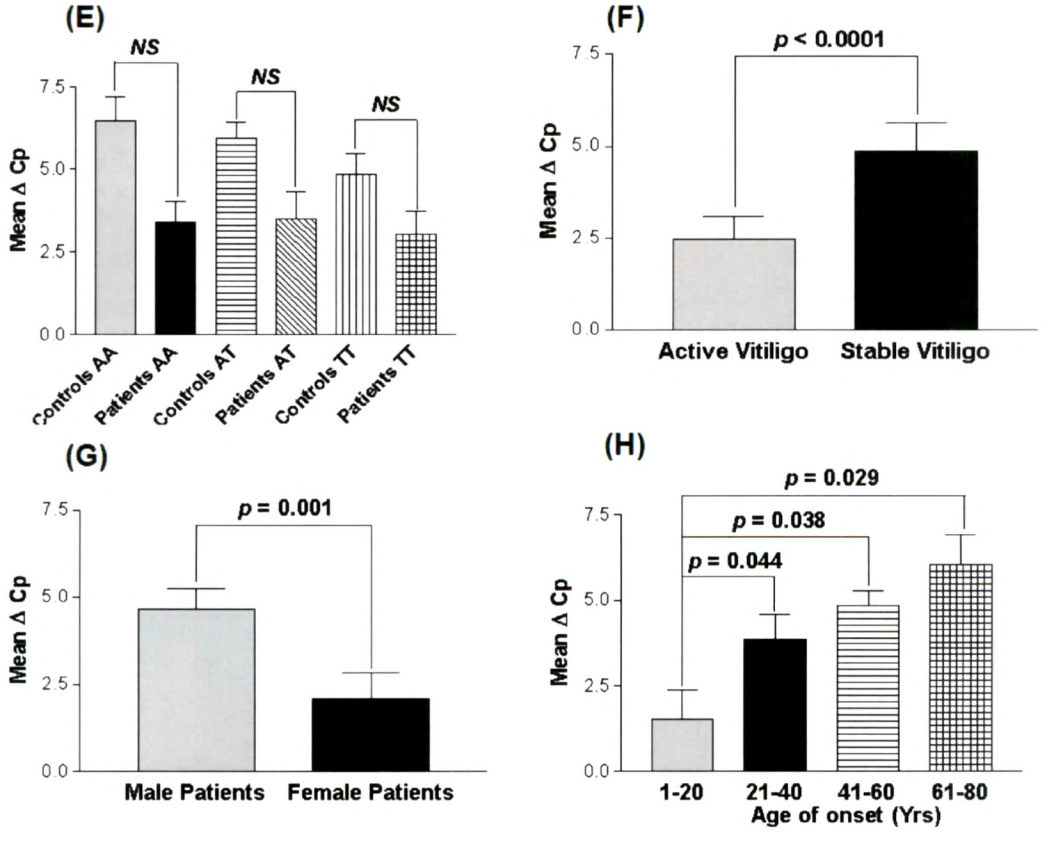
Figure 4. Relative gene expression of *NALP1* in controls and generalized vitiligo patients:

(A) Expression of *NALP1* transcripts in 175 controls, 122 generalized vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *NALP1* as compared to controls (Mean ΔCp ± SEM: 3.360 ± 0.5046 vs 6.276 ± 0.4784; p<0.0001).

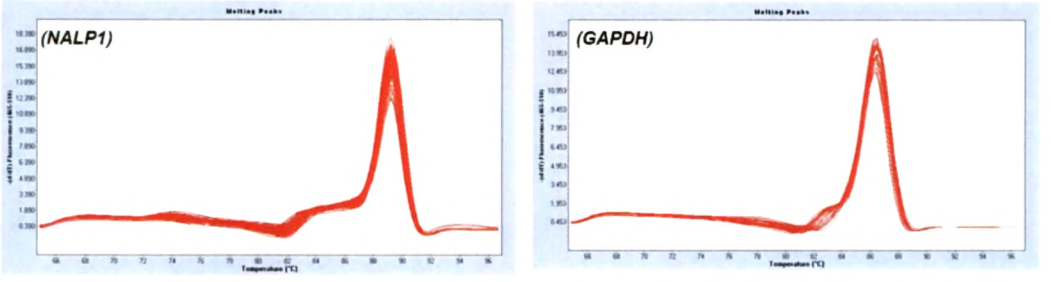
(B) Expression fold change of *NALP1* transcripts in 122 vitiligo patients against 175 controls showed 0.132 fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Expression of *NALP1* transcripts with respect to *NALP1* A/G (rs2670660) promoter polymorphism in 122 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *NALP1* with GG (Mean ΔCp ± SEM: 2.841 ± 0.7811 vs 5.796 ± 0.3632; p=0.001) and AG (Mean ΔCp ± SEM: 3.348 ± 1.000 vs 6.065 ± 0.3999; p=0.007) genotypes as compared to controls. There was no significant difference in the expression of *NALP1* in patients with AA genotypes (Mean ΔCp ± SEM: 5.357 ± 0.6911 vs 6.712 ± 0.4496; p=0.102) as compared to controls. [*NS* = non-significant]

(D) Expression of *NALP1* transcripts with respect to *NALP1* T/C (rs6502867) intron polymorphism in 122 vitiligo patients and 175 controls, as suggested by Mean ΔCp. Vitiligo patients showed significantly increased mRNA levels of *NALP1* with CC (Mean ΔCp ± SEM: 2.598 ± 0.9118 vs 5.434 ± 0.4912; p=0.020) and TC (Mean ΔCp ± SEM: 3.622 ± 0.6535 vs 6.125 ± 0.5546; p=0.006) genotypes as compared to controls. There was no significant difference in the expression of *NALP1* in patients with TT genotypes (Mean ΔCp ± SEM: 4.671 ± 1.079 vs 6.639 ± 0.6960; p=0.121) as compared to controls.



(I)



(E) Expression of *NALP1* transcripts with respect to *NALP1* A/T (rs12150220) intron polymorphism in 122 vitiligo patients and 175 controls, as suggested by Mean ΔCp. There was no significant difference in mRNA levels of *NALP1* in patients with TT (Mean ΔCp ± SEM: 3.040 ± 0.6779 vs 4.854 ± 0.6088; p=0.082), AT (Mean ΔCp ± SEM: 4.153 ± 1.025 vs 5.931 ± 0.4993; p=0.162) and AA (Mean ΔCp ± SEM: 4.753 ± 0.9490 vs 6.456 ± 0.7339; p=0.160) genotypes as compared to controls.

(F) Expression of *NALP1* transcripts with respect to activity of the disease in 91 patients with active vitiligo and 31 patients with stable vitiligo, as suggested by Mean ΔCp. Active vitiligo patients showed significantly increased mRNA levels of *NALP1* as compared to stable vitiligo patients (Mean ΔCp ± SEM: 2.472 ± 0.6268 vs 4.866 ± 0.7669; p=0.021).

(G) Expression of *NALP1* transcripts with respect to gender differences in 52 male patients and 70 female patients with vitiligo, as suggested by Mean ΔCp. Female patients with vitiligo showed significantly increased mRNA levels of *NALP1* as compared to male vitiligo patients (Mean ΔCp ± SEM: 4.649 ± 0.5885 vs 2.071 ± 0.7604; p=0.001).

(H) Expression of *NALP1* transcripts with respect to different age groups in 122 vitiligo patients, as suggested by Mean ΔCp. Vitiligo patients with the age of onset 1-20 yrs showed significantly increased expression of *NALP1* mRNA as compared to the age groups 21-40 yrs (Mean ΔCp ± SEM: 1.516 ± 0.8508 vs 3.853 ± 0.7290; p=0.044), 41-60 (Mean ΔCp ± SEM: 1.516 ± 0.8508 vs 4.841 ± 0.4153; p=0.038) and 61-80 yrs (Mean ΔCp ± SEM: 1.516 ± 0.8508 vs 6.028 ± 0.8713; p=0.029).

(I) Melt curve analysis of *NALP1* and *GAPDH* showing specific amplification.

7.3.6.2 Effect of *NALP1* expression on disease progression:

In addition, we also checked the effect of *NALP1* expression on progression of the disease i.e. active and stable cases (Figure 4F). Interestingly, active vitiligo patients showed significant increase in expression of *NALP1* transcripts as compared to the patients with stable vitiligo (p=0. 021) suggesting the involvement of *NALP1* 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 *NALP1* expression as compared to male patients (p=0. 001) (Figure 4G).

When *NALP1* expression was monitored in different age of onset groups of patients, patients with the age group 1-20 yrs showed significantly increased expression of *NALP1* transcripts as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.044, p=0.038 and p=0.029 respectively) suggesting the importance of *NALP1* in early onset of the disease (Figure 4H).

7.4 DISCUSSION

The locus for familial vitiligo on chromosome 17 harbours the gene coding for *NALP1* (Jin *et al.*, 2007a). The protein contains a NACHT domain, LRR domain (leucine rich repeat) and a pyrin domain. NALP1 protein is a component of a large signal induced cytoplasmic multiprotein complex called inflammasome, which is a direct sensor of bacterial infections (Kummer *et al.*, 2007). NALP1 contains a caspase recruitment domain which is known to be the key mediator of apoptosis. The N-terminal pyrin like motif is involved in protein-protein interaction which interacts strongly with caspase-2 caspase-9 and apoptotic protease activating factor-1 (Apaf-1) (Faustin *et al.*, 2007; Bonfoco *et al.*, 2001).

NALP1 is known to be involved in inflammation and apoptosis (Martinon *et al.*, 2001; Tschopp *et al.*, 2003). NALP1 may also contribute to modulate the response of cells towards proinflammatory stimuli like IL-1β, IFN-γ and TNF-α. Overexpression of this gene induces apoptosis in cells (Kummer *et al.*, 2007). Even transient overexpression of *NALP1* in *in vitro* cultured cells was sufficient to induce apoptosis. The primary function of human NALP1 is caspase activation. Upon activation by

appropriate pathogen associated molecular pattern (PAMP)s, resulting in its oligomerization, human NLRP1 binds adapter protein apoptosis associated speck like protein containing CARD (ASC) via its N-terminal PYRIN-PAAD-DAPIN domain (PYD). ASC, in turn, binds pro-caspase-1, forming the inflammasome (Martinon *et al.*, 2002). The C-terminal CARD of NALP1 can also bind both pro-caspase-1 and pro-caspase-5 (Tschopp *et al.*, 2003). These members of the caspase family activate IL-1β and IL-18, as well as cause apoptosis.

NALP1 may have other functions besides activation of caspase-1 in response to bacterial PAMP, muramyl dipeptide (MDP). For example, NLRP1 was reported to bind the patched receptor complex via adapter protein DRAL, stimulating caspase-9 activation (Mille *et al.*, 2009). In this regard, the CARD of NALP1 binds the CARD of Apaf-1, a caspase-9 activating protein that becomes activated by cytochrome c when released from mitochondria (Bruey *et al.*, 2007; Chu *et al.*, 2001). Thus, it is possible that NALP1 contributes to apoptosis via caspase-9 activation in some contexts. Also, it is possible that Apaf1 and components of patched receptor complex are intrinsic activators of NALP1. Cytochrome c-mediated oligomerization of Apaf-1 conceivably could create a platform for recruitment of NALP1, resulting in caspase-1 activation via a mechanism whereby the N-terminal PYD domain of NALP1 binds ASC, which in turn binds pro-caspase-1, while the C-terminal CARD of NRLP1 binds the CARD of Apaf1. Activated patched receptors also could theoretically create an oligomerized protein complex at the plasma membrane for NALP1 recruitment, thus promoting caspase activation.

NALP1 is widely expressed at low levels in all cells but is expressed at high levels in blood mononuclear cells and immune cells, particularly granulocytes, monocytes, T-lymphocytes and Langerhans cells. Immunohistochemical studies have found high *NALP1* expression in epidermis within Langerhans cells (Kummer *et al.*, 2007) suggesting involvement of NALP1 in skin autoimmunity. NALP1 is part of inflammasomes that regulate the activation of caspases which in turn convert proinflammatory cytokines into their active forms. Taieb (2007) suggested that NALP1 could help trigger or enhance the autoimmune part of vitiligo, which may involve initial contacts between melanocytes and NALP1-positive Langerhans.

Earlier, several SNPs in *NALP1* have been studied with vitiligo susceptibility; however, only a few of them achieved significant association with vitiligo (Jin *et al.*, 2007a,b; Alkhateeb, 2010). The functional significance of these SNPs is not yet known, but they are proposed to have a regulatory effect on *NALP1* expression. The A/G (rs2670660) SNP is present in *NALP1* promoter region which is conserved in human, chimpanzee, macaque, bush baby, cow, mouse and rat, suggesting that this variant is functionally significant. It alters predicted binding motifs for the transcription factors high mobility group protein (HMGA1) [HMG-I(Y)] and MYB. MYB regulates transcription during the differentiation, proliferation, and apoptosis of erythroid, myeloid, and lymphoid cell lineages. The structural polymorphism A/T (rs12150220) is a non-synonymous coding SNP which substitutes Leucine at 155 position toHistidine. The amino acid sequence, including Leu155, is highly conserved throughout primate evolution, suggesting that this region is critical for protein function. The other T/C (rs6502867) SNP is located in an intron towards the 3' end of the *NALP1* gene.

Variations in the *NALP1* gene have been reported to confer risk for vitiligo and extended autoimmune disorders in Caucasian patients from the United Kingdom, the United States and Romania (Jin *et al.*, 2007a, b). This study confirms genetic association of generalized vitiligo with variation in *NALP1*, which contains at least two independent risk signals, one tagged by SNP rs2670660 and another tagged by SNPs rs2670660 and rs8182352. A report from cohort of Arab vitiligo patients suggested significant association of two SNPs in the *NALP1* extended promoter region: rs1008588 and rs2670660 with generalized vitiligo (Alkhateeb and Qarqaz, 2010). Our present study focuses on identification of genetic variants of *NALP1* which were significantly associated with vitilgo susceptibility in earlier studies. Intersetingly, we also found significant association of *NALP1* rs2670660 (A/G) and rs6502867 (T/C) SNPs with vitiligo susceptibility in Gujarat population; however rs12150220 (A/T) polymorphism was not associated with vitiligo susceptibility (Table 3). In particular, the 'G' allele of promoter polymorphism (A/G; rs2670660) which may be involved in regulation of *NALP1* expression was found to be prevalent in vitiligo especially in active cases of vitiligo which signifies that NALP1 also modulates the disease preogression. Moreover, the minor allele 'G' was prevalent in female patients compared to male patients suggesting the inclination of autoimmune

preponderance in females (Table 5). Another SNP rs2670660 (T/C) present in the intronic region of *NALP1* was also found to be in significant association with generalized vitiligo, active cases of vitiligo and with female vitiligo patients (Table 3, 4 & 5).

The present study found significant increase in expression of *NALP1* transcripts in vitiligo patients (Figure 4A). The expression of *NALP1* was also increased in active cases of vitiligo as compared to patients with stable vitiligo (Figure 4F). Also, female patients had increased expression of *NALP1* as compared to male patients with vitiligo suggesting that females are more inclined to autoimmunity (Figure 4G). The *NALP1* expression was also increased in patients with early onset of vitiligo as compared to those of late onset suggesting the important role of NALP1 in early phase of the disease (Figure H). In support of this, age of onset analysis with different genotypes of *NALP1* polymorphisms such as A/G (rs2670660) and T/C (rs6502867) revealed that patients harboring susceptible genotypes had an early onset of vitiligo as compared to those with wild type genotypes (Figure 3A & B).

Furthermore, the genotype-phenotype correlation for *NALP1* A/G (rs2670660) SNP revealed increased expression of *NALP1* in patients with GG and AG genotypes as compared to those of controls indicating that 'G' allele may be involved in the increased expression of *NALP1* (Figure 4C). Moreover, genotype-phenotype analysis of *NALP1* T/C (rs6502867) SNP showed increased expression of *NALP1* in patients with CC and TC genotypes as compared to those of controls, suggesting the important role of 'C' allele in increased expression of *NALP1* (Figure 4D). However, the non-synonymous A/T (rs12150220) SNP did not reveal any significant correlation of its genotype with the *NALP1* mRNA expression (Figure 4E). Furthermore, the frequency of susceptible haplotype 'GCT' was significantly increased in vitiligo patients as compared to controls suggesting the important role of *NALP1* polymorphisms in susceptibility of generalized vitiligo.

Nevertheless, the exact functional consequences of these polymorphic variants remain to be determined. The complexity of NALP1 regulation and its various putative effector mechanisms (inflammation, apoptosis, etc.) raise several interesting possibilities for how the sequence variations in NALP1 might impact immune system

function. The current study attempted to elucidate the role of these variants; however, further investigations are needed to elaborate the mechanistic insights that might reveal functional consequences of *NALP1* variants in vitiligo and other autoimmune disorders. In conclusion, our findings suggest that the increased *NALP1* expression in vitiligo patients could result, at least in part, from variations at the genetic level. The study also emphasizes the influence of *NALP1* on the disease progression, onset of the disease and gender biasness for developing vitiligo. More detailed studies regarding the role of *NALP1* in precipitation of vitiligo may prove to be useful for development of effective preventive/ameliorative therapies.

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