CHAPTER V

CYTOTOXIC T-LYMPHOCYTE ASSOCIATED ANTIGEN-4 (*CTLA*-4) GENE POLYMORPHISMS AND THEIR GENOTYPE-PHENOTYPE CORRELATION WITH GENERALIZED VITILIGO SUSCEPTIBILITY

5.1 INTRODUCTION

Vitiligo is an acquired, hypomelanotic disease characterized by circumscribed depigmented macules. The 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, 1993). In India, the incidence of vitiligo is found to be 0.5% (Das *et al.,* 1985). The autoimmune destruction of melanocytes can be explained by the abnormalities in both humoral and cell-mediated immunity (Shajil *et al.,* 2006a; Kemp *et al.,* 2001). The autoimmune hypothesis gains further support from immunotherapy studies of melanoma patients (Rosenberg, 1997). Vitiligo is a polygenic disease and several candidate genes including the major histocompatibility complex (*MHC)*, angiotensin converting enzyme (*ACE)*, catalase (*CAT)*, cytotoxic T-lymphocyte associated antigen-4 (*CTLA4*), catechol o-methyl transferase (*COMT)*, estrogen receptor (*ESR)*, mannose-binding lectin 2 (*MBL2*) and protein tyrosine phosphatase non-receptor type-22 (*PTPN22*) that play a role in regulating immunity have been tested for genetic association with generalized vitiligo (Spritz, 2007; Spritz, 2008).

Recent studies postulate that the peripheral mechanisms of T cell tolerance are essential to control self-reactive T cells and the elimination of self-tolerance may result in the development of autoimmune diseases (Bluestone, 1997). Hence, molecules affecting these mechanisms are obvious candidates for conferring risk to autoimmunity.

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4; CD152) is expressed exclusively on activated CD4+ and CD8+ T cells and binds the same ligands, B7-1 and B7-2, as CD28 but with a 20 to 50 fold higher affinity (Walunas *et al.,* 1994). However, CD28 provides a critical costimulatory signal essential for the initiation and progression of T cell immunity, data indicate that *CTLA*-4 may actually function to down-regulate T cell function (Lenschow *et al.,* 1996; Walunas *et al.,* 1994). The CTLA-4:B7 interaction plays a critical role in regulating self-tolerance, and hence in susceptibility to autoimmune diseases. The human *CTLA4* gene maps to chromosome 2q33 and comprises 3 exons in addition to the leader sequence. The human *CTLA4*

sequence encodes two transcripts of 1.8 and 0.8 kb, respectively known as full length *CTLA4* (*flCTLA4*) and soluble *CTLA4* (*sCTLA4*) (Harper *et al.,* 1991).

The single nucleotide polymorphism: A to G transition at position 49 (A49G) of exon 1 leads to an alanine to threonine amino acid substitution at codon 17 of the leader peptide (A17T) (Nistico *et al.,* 1996). The +49A/G, is in linkage disequilibrium with CT60 in Poles and other Caucasians (Holopainen *et al.,* 2001; Ueda *et al.,* 2003). A few studies have addressed the possible functional significance of these polymorphisms of *CTLA4*. Interestingly, the G allele of *CTLA4* +49A/G SNP was reported to be involved in the altered intracellular transport of the CTLA-4 protein and its availability on the cell surface. In addition, the 3' UTR CT60A/G allelic variation was reported to be correlated with lower mRNA levels of the soluble alternative splice form of *CTLA4* (*sCTLA4*), suggesting its crucial role in autoimmune diseases (Ueda *et al.,* 2003). One report suggested a correlation between the exon 1 +49A/G SNP and *CTLA4* mRNA levels in human peripheral blood mononuclear cells, whereas another study was unable to demonstrate any significant correlation (Barnes *et al.,* 1997; Cavallo *et al.,* 1997). However, both the studies were preliminary and based on relatively few samples, thus further studies addressing this aspect are warranted.

Using the candidate gene approach, the role of *CTLA4* has been investigated in various autoimmune diseases e.g. Graves disease, Hashimoto's thyroiditis (HT), Addison's disease (AD), Insulin-dependent diabetes mellitus (IDDM), Myasthenia gravis (MG), Multiple sclerosis (MS), Systemic lupus erythematosus (SLE), Rheumatoid arthritis (RA) including Vitiligo. As CTLA-4 appears to be a negative regulator of the normal immune response, it is of interest to investigate the genotype-phenotype correlation of *CTLA4* in vitiligo. Hence, the aims of this study were: i.) to measure and compare *CTLA4* mRNA levels in patients with vitiligo and in unaffected controls; ii.) to determine whether the two well-characterized *CTLA4* polymorphisms [exon1 +49AG (rs231775) and 3' UTR CT60A/G (rs3087243)] are associated with vitiligo susceptibility and modulate *CTLA4* mRNA levels in these groups.

5.2 MATERIALS AND METHODS

5.2.1 Study Subjects:

The study group included 437 vitiligo patients comprised of 190 males and 247 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. The study involved generalized vitiligo patients including acrofacial vitiligo and vitiligo universalis. A total of 746 ethnically and sex-matched unaffected individuals were included as controls in this study. The control group comprised 331 males and 415 females (Table 1). None of the healthy individuals had any evidence of vitiligo and any other autoimmune diseases.

The study plan was approved by the ethical committee of Department of Biochemistry, 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 Patients	**Controls**
	(n =437)	(n =746)
Average age (mean age ± SD)	32.45 ± 13.48 yrs	28.23 ± 14.42yrs
Sex: male	190 (43.48%)	331 (44.37%)
female	247 (56.52%)	415 (55.63%)
Onset age (mean age ± SD)	21.25 ± 12.53 yrs	NA
Duration of disease (mean ± SD)	7.8 ± 6.9 yr	NA
Family history	57 (13.04%)	NA

5.2.2 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.

5.2.3 Determination of *CTLA4* gene polymorphisms:

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used to genotype exon 1 +49A/G and 3' UTR CT60A/G polymorphisms of *CTLA4* gene. 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 30 cycles of 95°C for 30 seconds, primer dependent annealing for 30 seconds (Table 2), 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) used were: *BstE*II and *Nco*I for digesting amplicons of exon 1 +49A/G and 3' UTR CT60A/G of *CTLA4* gene. 5 µL of the amplified products were digested for 16 hours at 37°C with 1 U of the corresponding restriction enzyme in a total reaction volume of 25 µL. The digestion products with 100 base pair DNA ladder (Bioron, Ludwigshafen am Rhein, Germany) were resolved in 3.5% agarose gels stained with ethidium bromide and visualized under UV transilluminator.

Table 2. Primers used for *CTLA4* SNPs genotyping and gene expression analysis.

Gene/SNP	Primer Sequence	Annealing Temperature	Amplicon size (bp)	Restriction Enzyme (Digested Products)
(rs231775)				
CTLA4 +49A/G F	5'-AAGGCTCAGCTGAACCTGGT-3'	60°C	271	*BstE*II (249 & 22 bp)
CTLA4 +49A/G R	5'-CTTTGCCTTATTTGCTGCCGC-3'			
(rs3087243)				*Nco*I
CTLA4 CT60A/G F	5'-CACCACTATTTGGGATATACC-3'	63°C	216	(174 & 42 bp)
CTLA4 CT60A/G R	5'-AGCTCTATATTTCAGGAAGGC-3'			
flCTLA-4 gene expression F	5'-TATGTAATTGATCCAGAACCGTGC3'	63°C	123	-
flCTLA4 gene expressionR	5'-TAGCATTTTGCTCAAAGAAACAG-3'			
sCTLA4 gene expression F	5'GAACCCAGATTTATGTAATTGCTAAG3'	63°C	88	-
sCTLA4 gene expressionR	5'-CACATTCTGGCTCTGTTGGG-3'			
GAPDH gene expression F	5'-ATCCCATCACCATCTTCCAGGA-3'	63°C	122	-
GAPDH gene expressionR	5'-CAAATGAGCCCCAGCCTTCT-3'			

5.2.4 Determination of s*CTLA*4, fl*CTLA*4 and *GAPDH* mRNA expression:

5.2.4.1 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/ ethidium bromide staining and O.D. 260/280 absorbance ratio >1.95. 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).

5.2.4.2 Real-time PCR:

The levels of full length, soluble *CTLA*4 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 (95°C for 15 s, 63°C for 30 s, 72°C for 30 s). The fluorescent data collection was performed during the extension step (Figure 7A). At the end of the amplification phase a melt curve analysis was carried out on the product formed (Figure 7B). The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

5.2.5 Statistical analysis:

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 the genotypes using chi-square analysis. The distribution of the

genotypes and allele frequencies of *CTLA*4 +49A/G and CT60A/G polymorphisms for patients and control subjects were compared using the chi-square test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003).

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). Differences were considered to be statistically significant if the *p*-value was ≤ 0.05. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Relative expression of both full length *CTLA*4 (flCTLA4) and soluble *CTLA*-4 (s*CTLA*4) 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).

5.3 RESULTS

5.3.1 Analysis of association between *CTLA*4 gene exon 1 +49A/G polymorphism and susceptibility to vitiligo:

PCR-RFLP for +49A/G polymorphism yielded a 271 bp undigested product corresponding to G allele and 249 bp and 22 bp digested products corresponding to A allele. The three genotypes identified by 3.5% agarose gel electrophoresis were: AA homozygous, AG heterozygous and GG homozygous for +49A/G polymorphism of *CTLA*4 gene (Figure 1A).

Exon 1 +49A/G polymorphism of *CTLA*4 gene was not found to be associated with vitiligo patients (p=0.771) when genotypes were compared with chi-square test-3x2 contingency table (Table 3). Also, there was no significant difference in allele frequencies of this polymorphism between patients and controls when compared with 2x2 contingency table (p=0.461) (Table 3). Both patient and control populations were found to be in Hardy-Weinberg equilibrium for this polymorphism (p=0.272 and p=0.126 respectively) (Table 3). This study has 95% statistical power for the effect

size 0.1 to detect association of +49A/G polymorphism of *CTLA*-4 at $p<0.05$ in patients and control population.

5.3.2 Analysis of association between 3' UTR *CTLA*4 gene CT60A/G polymorphism and susceptibility to vitiligo:

The genotyping of CT60A/G polymorphism revealed a 216 bp undigested product corresponding to G allele and 174 bp and 42 bp digested products corresponding to A allele by PCR-RFLP method. The three genotypes identified by 3.5% agarose gel electrophoresis were: AA homozygous, AG heterozygous and GG homozygous for CT60A/G polymorphism of *CTLA*4 gene (Figure 1B).

The 3' UTR CT60A/G polymorphism of *CTLA*4 gene was found to be in significant association with vitiligo patients ($p=0.0002$) when genotypes were compared using chi-squared test-3x2 contingency table with Bonferroni's correction (Table 3). Also, there was significant difference in allele frequencies of this polymorphism between patients and controls when compared with 2x2 contingency table ($p=0.0002$) (Table 3). Control population was found to be in Hardy-Weinberg equilibrium for this polymorphism however patient population deviated from the equilibrium ($p=0.075$ and $p<0.0001$ respectively) (Table 3). This study has 96% statistical power for the effect size 0.1 to detect association of CT60A/G polymorphism of *CTLA*4 at $p<0.05$ in patients and control population.

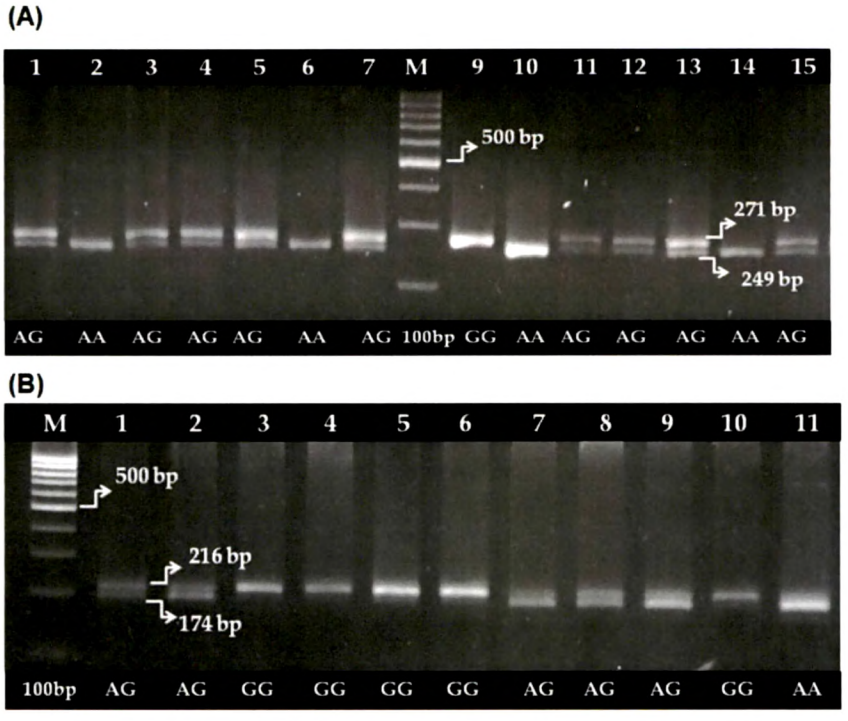


Figure 1. PCR-RFLP analysis of *CTLA*4 exon 1 +49 A/G and3' UTR CT60A/G polymorphisms:

(A) PCR-RFLP analysis of *CTLA*4 exon 1 +49 A/G polymorphism on 3.5 % agarose gel electrophoresis: lanes: 1, 3, 4, 5, 7, 11, 12, 13 & 15 show heterozygous (AG) genotypes; lanes: 2, 6, 10 & 14 show homozygous (AA) genotypes; lane: 9 shows homozygous (GG) genotype; lane M shows 100 bp DNA ladder.

(B) PCR-RFLP analysis of *CTLA*4 3' UTR CT60A/G polymorphism on 3.5 % agarose gel electrophoresis: lanes: 1, 2, 7, 8 & 9 show heterozygous (AG) genotypes; lanes: 3, 4, 5, 6, & 10 show homozygous (GG) genotypes; lane: 11 shows homozygous (AA) genotype; lane M shows 100 bp DNA ladder.

Table 3. Association studies for *CTLA4* gene exon 1 +49A/G and 3' UTR CT60A/G polymorphisms in generalized vitiligo patients.

SNP	Genotype or allele	Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n =347)	(n =746)			
	AA	169 (0.49)	347 (0.47)			
rs231775	AG	140 (0.40)	310 (0.41)	0.771		
Exon 1	GG	38 (0.11)	89 (0.12)		0.272	
(+49A/G)	Allele				(P)	0.930
	A	478 (0.69)	1004 (0.67)	0.461		(0.766-
	G	216 (0.31)	488 (0.33)		0.126	1.128)
					(C)	
	Genotype	(n = 437)	(n = 738)			
	AA	146 (0.33)	256 (0.35)			
rs3087243	AG	135 (0.31)	337 (0.46)	0.0002*		
3' UTR	GG	156 (0.36)	145 (0.19)		<0.0001	
(CT60A/G)	Allele				(P)	1.417
	A	427 (0.49)	849 (0.58)	0.0002*	0.075	(1.198-
	G	447 (0.51)	627 (0.42)		(C)	1.677)

'n' represents number of Patients/ Controls,

HWE refers to Hardy-Weinberg Equilibrium,

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

* Bonferroni's corrected *p* value.

5.3.3 Linkage disequilibrium (LD) and haplotype analyses:

The LD analysis revealed that the two polymorphisms investigated in the *CTLA4* gene were in moderate LD association (+49A/G: CT60A/G; D' =0.64, r^2=0.11). A haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes did not differ between vitiligo patients and controls (global *p*-value = 0.123) (Table 4). However, the GG haplotype was more frequently observed in vitiligo patients and increased the risk of vitiligo by 1.2-fold [*p* = 0.048; odds ratio (OR): 1.243; 95% confidence interval (CI): (1.001-1.543)] (Table 4).

Table 4. Distribution of haplotypes frequencies for *CTLA4* gene polymorphisms (+49A/G and CT60A/G) among generalized vitiligo patients and controls.

Haplotype (+49A/G and CT60A/G)	Patients (Freq. %) (n=654)	Controls (Freq. %) (n=1374)	*p* for Association	$p_{(global)}$	Odds ratio (95% CI)
AA	178.29 (27.3)	388.84 (28.3)	0.660		0.951 (0.758-1.191)
AG	218.71 (33.4)	523.49 (38.1)	0.061	0.123	0.817 (0.661-1.009)
GA	30.71 (4.7)	52.21 (3.8)	0.372		1.256 (0.761-2.071)
GG	226.29 (34.6)	10.83(29.8)	0.048		1.243 (1.001-1.543)

5.3.4 The expression of fl*CTLA4* and s*CTLA4* transcripts:

Comparison of the findings shows significantly decreased expression of both full length and soluble *CTLA4* in vitiligo patients than in unaffected controls after normalization with *GAPDH* expression (p=0.007 and p=0.037 respectively) (Figure 2A). The $2^{-\Delta\Delta Cp}$ analysis showed approximately two fold change in the expression of fl*CTLA4* and s*CTLA4* mRNA expression in patients as compared to controls (Figure 2B).

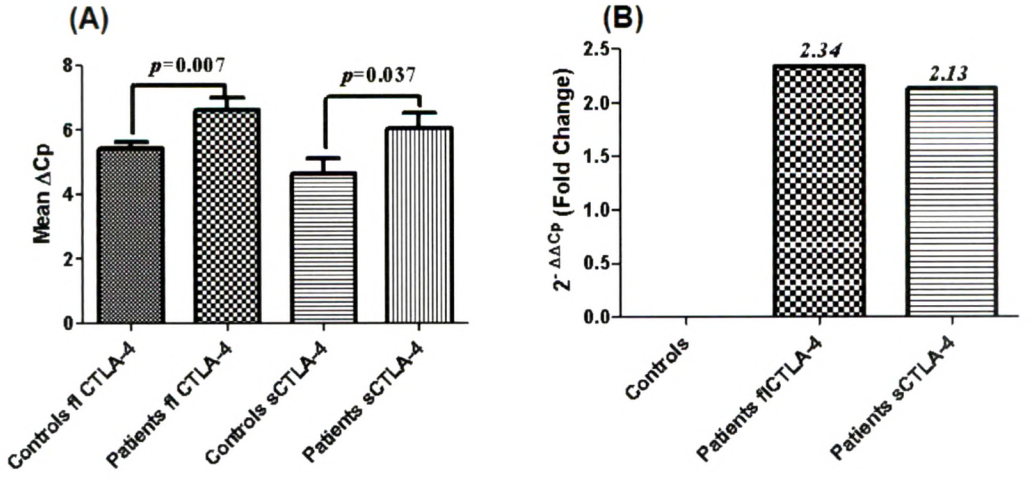


Figure 2. Relative gene expression of fl*CTLA*4 and s*CTLA*4 in controls and vitiligo patients:

(A) Expression of fl*CTLA*4 and s*CTLA*4 mRNA in 76 vitiligo patients and 83 controls as suggested by Mean ΔCp. Vitiligo patients showed significantly reduced mRNA levels of fl*CTLA*4 (p=0.007) and s*CTLA*4 (p=0.037) as compared to controls.

(B) Expression fold change of fl*CTLA*4 and s*CTLA*4 in 76 vitiligo patients against 83 controls showed approximately two fold change as determined by $2^{-\Delta\Delta Cp}$ method.

5.3.5 Genotype-phenotype correlations for s*CTLA*4 and fl*CTLA*4 in vitiligo patients and controls:

We analyzed the mRNA expression of fl*CTLA*4, s*CTLA*4 based on the +49A/G and CT60A/G genotypes of 76 vitiligo patients and 83controls. The expression levels of fl*CTLA*4 for AA, AG and GG genotypes of exon 1 +49A/G polymorphism did not differ significantly in vitiligo patients as compared to controls (p=0.122, p=0.320 and p=0.068 respectively) (Figure 3A). Also, s*CTLA*4 expression did not differ for AA, AG and GG genotypes of exon 1 +49A/G polymorphism in vitiligo patients as compared to controls (p=0.877, p=0.437 and p=0.360 respectively) (Figure 3B). However, the expression levels of both fl*CTLA*4 and s*CTLA*4 were differed significantly for GG genotype of CT60A/G polymorphism in vitiligo patients as compared to controls (p=0.004 and p=0.005 respectively) (Figure 4A & 4B). The AA genotype did not differ for fl*CTLA*4 and s*CTLA*4 expression levels in patients and controls (p=0.343 and p=0.205 respectively) (Figure 4A & 4B).

Further, the expression levels of both fl*CTLA*4 and s*CTLA*4 were analyzed with respect to haplotypes generated from the two investigated polymorphisms of *CTLA*4 (Fig. 4A & 4B). Both fl*CTLA*4 and s*CTLA*4 expression levels were significantly differed and associated with AG haplotypes in patients and controls (p=0.036 and p=0.020 respectively). Other, three haplotypes: AA, GA and GG did not differ with respect to fl*CTLA*4 and s*CTLA*4 expression levels in patients and controls (p=0.955 & p=0.152, p=0.476 & p=0.865, p=0.075 & p=0.992 respectively).

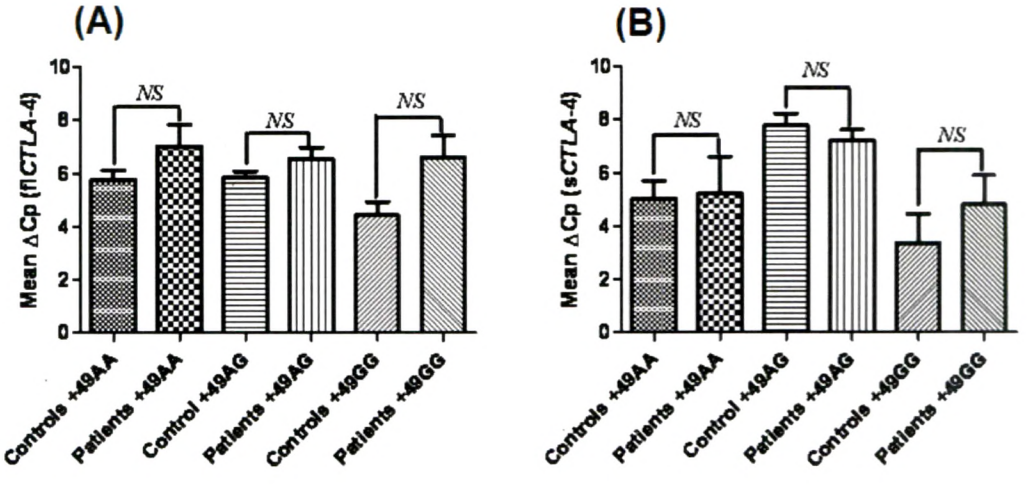


Figure 3. Genotype - phenotype correlation of exon 1 +49A/G polymorphism of fl*CTLA*4 and s*CTL*A4 in controls and vitiligo patients:

(A) Relative mRNA expression of *flCTLA4* with respect to +49A/G genotypes in 76 patients and 83 controls. None of the three genotypes AA (p=0.122), AG (p=0.320) and GG (p=0.068) in patients showed significant difference for *flCTLA*4 expression as compared to controls a suggested by Mean ΔCp.

(B) Relative mRNA expression of *sCTLA4* with respect to +49A/G genotypes in 76 patients and 83 controls. None of the three genotypes AA (p=0.877), AG (p=0.437) and GG (p=0.360) in patients showed significant difference for *flCTLA4* expression as compared to controls a suggested by Mean ΔCp.

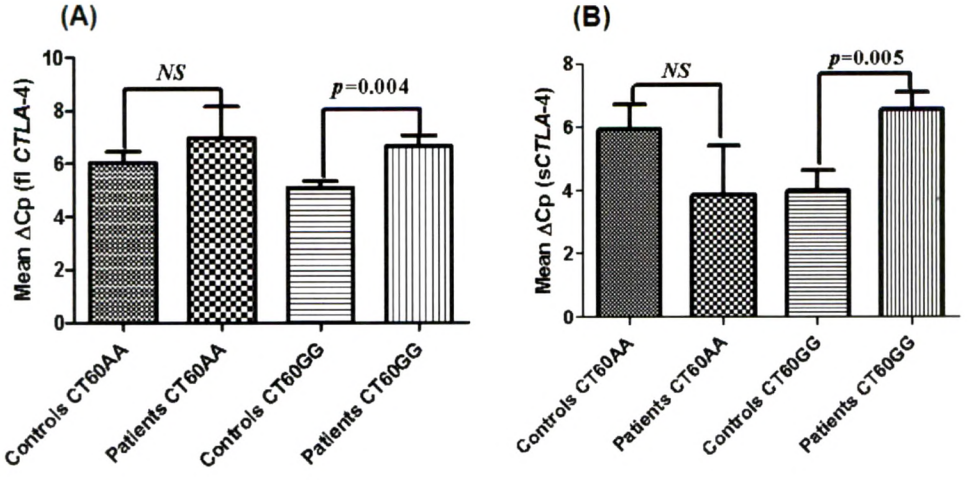


Figure 4. Genotype - phenotype correlation of 3' UTR CT60A/G polymorphism of *flCTLA4* and *sCTLA4* in controls and vitiligo patients:

(A) Relative mRNA expression of *flCTLA4* with respect to CT60A/G genotypes in 76 patients and 83 controls. GG genotype showed significant decrease in the levels of *flCTLA4* mRNA (p=0.004) in patients as compared to AA genotype (p=0.343) as suggested by Mean ΔCp.

(B) Relative mRNA expression of *sCTLA4* with respect to CT60A/G genotypes in 76 patients and 83 controls. GG genotype showed significant decrease in levels of *sCTLA4* mRNA (p=0.005) in patients as compared to AA genotype (p=0.205) as suggested by Mean ΔCp. [NS = non-significant]

5.3.6 Ratio of *sCTLA4* and *flCTLA4* mRNA expression in vitiligo patients and controls:

The expression level of *sCTLA4* and *flCTLA4* was also analyzed as ratio of *sCTLA4*: *flCTLA4* in vitiligo patients and controls. We could not detect any significant difference in the ratio of *sCTLA4* to *flCTLA4* mRNA expression between patients and controls (p=0.346) (Figure 5A). However, AG genotype of exon 1 +49A/G polymorphism showed significant difference for the ratio of *sCTLA4* and *flCTLA4* mRNA expression in patients and controls (p=0.049) (Figure 6A). Whereas the other two genotypes: AA and GG did not differ for the ratio of *sCTLA4* and *flCTLA4* mRNA expression in patients and controls (p=0.668 and p=0.964) (Figure 6A). When ratio of *sCTLA4* and *flCTLA4* mRNA expression for CT60A/G genotypes were

compared in patients and controls GG genotype showed significantly decreased ratio (p=0.019), conversely the AA genotype did not differ significantly (p=0.096) (Figure 6B). Moreover, ratio of *sCTLA4* and *flCTLA4* mRNA expression did not differ significantly for any of the haplotypes (AA, AG, GA, GG) in patients and controls (p=0.159, p=0.068, p=0.966 and p=0.585 respectively) (Figure 5B).

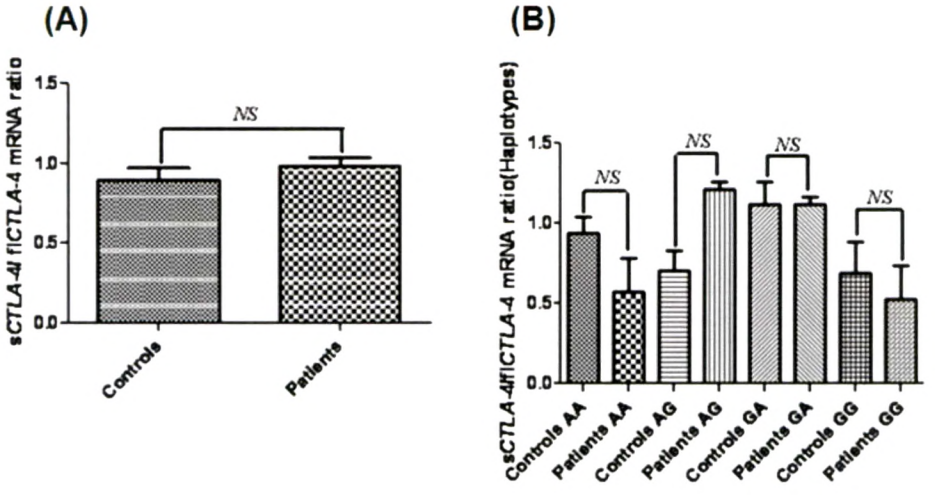


Figure 5. Expression of human *sCTLA4* and *flCTLA4* mRNA ratio in controls and vitiligo patients:

(A) *sCTLA4/flCTLA4* mRNA ratio was measured in 76 vitiligo patients and 83 unaffected controls which was not found altered (p=0.346).

(B) *sCTLA4/flCTLA4* mRNA ratio was analyzed with respect to (+49A/G: CT60A/G) haplotypes in patients and controls. None of the haplotypes showed significant difference in the *sCTLA4/flCTLA4* mRNA ratio (AA, AG, GA, GG) in patients and controls (p=0.159, p=0.068, p=0.966 and p=0.585 respectively). [*NS* = non-significant]

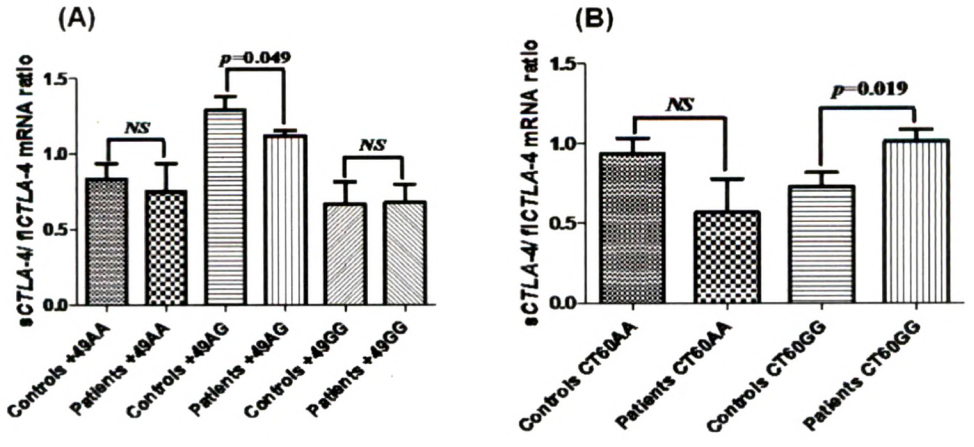
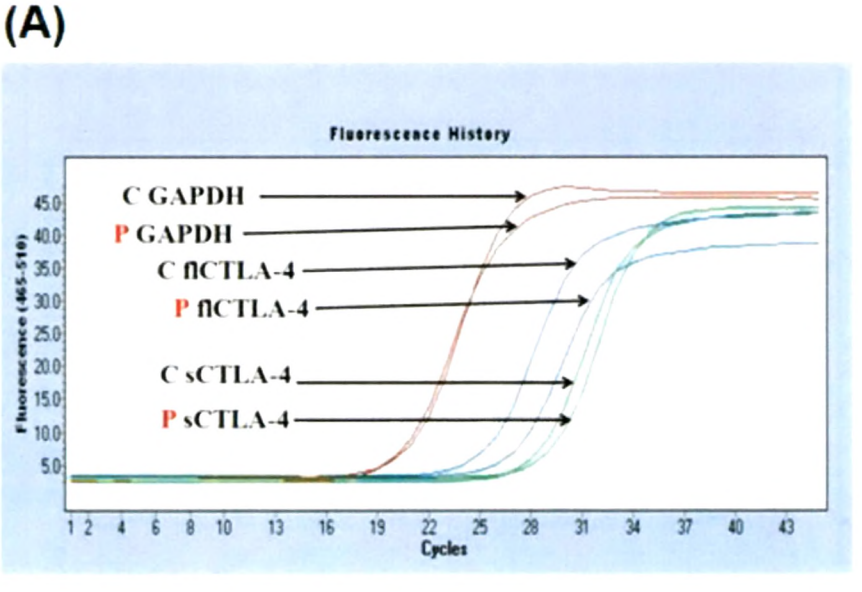


Figure 6. Expression of human *sCTLA4* and *flCTLA4* mRNA ratio with respect to +49A/G and CT60A/G genotypes in controls and patients:

(A) *sCTLA4/flCTLA4* mRNA ratio was analyzed with respect to +49A/G genotypes in patients and controls. AG genotype showed significant decrease in the ratio (p=0.049) as compared to AA (p= 0.668) and GG (p=0.964) genotypes.

(B) s*CTLA4/flCTLA4* mRNA ratio was analyzed with respect to CT60A/G genotypes in patients and controls. GG genotype showed significant increase in the ratio (p=0.019) as compared to AA (p=0.096) genotype.



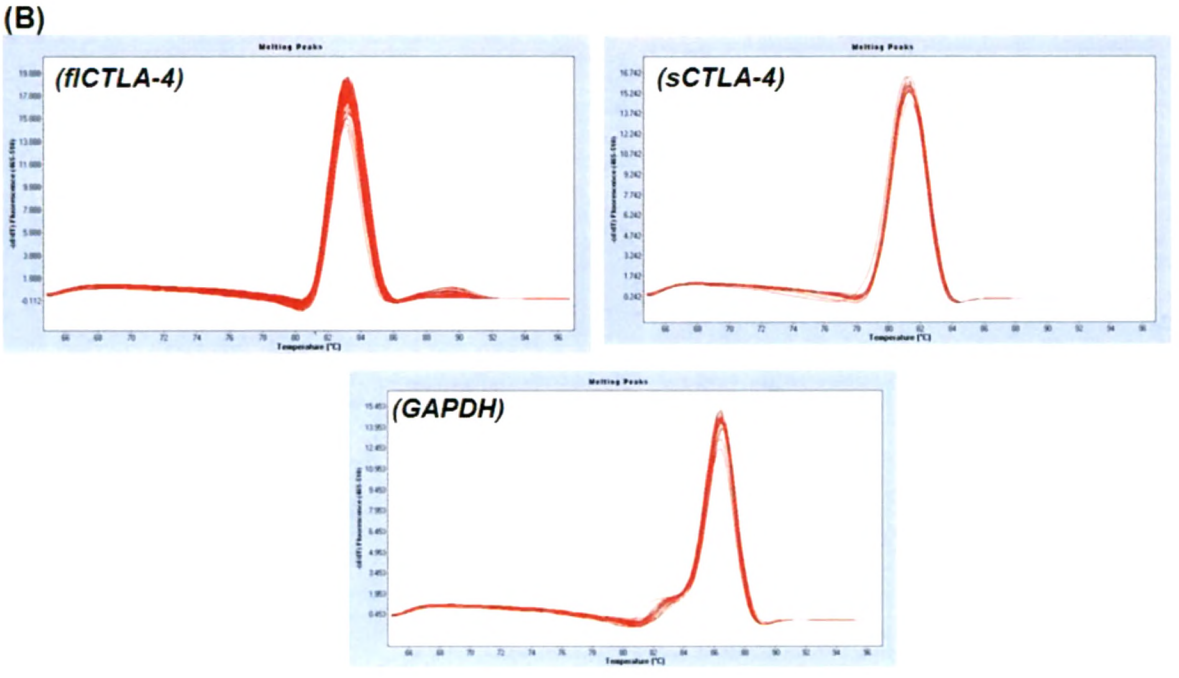


Figure 7. Amplification curve for relative gene expression of *flCTLA4* and *sCTLA4*:

(A) Representative amplification curve for *flCTLA*4 and *sCTLA4* with *GAPDH* as reference gene. 'C' refers to Control and 'P' refers to Patient. **(B)** Melt curve analysis of *flCTLA4*, *sCTLA4* and *GAPDH* showing specific amplification.

5.4 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). Attempts to identify genes involved in susceptibility to vitiligo have involved gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses to discover new genes. Autoimmunity has been suggested to play a major role in the pathogenesis of vitiligo since it shows frequent association with other autoimmune disorders such as rheumatoid arthritis, Graves' disease etc. (Ochi *et al.,* 1969). 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). Our study also suggested that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil *et al.,* 2006b). Also, in the present study 13.04% of vitiligo patients have one or more first degree relative affected suggesting the involvement of genetic factors in pathogenesis of vitiligo.

CTLA-4 is an important molecule in the down-regulation of T-cell activation and it is critical for the activity of regulatory T cells. The CTLA4 gene produces two different isoforms: a full length protein (flCTLA4) encoded by exon 1 (peptide leader), exon 2 (extracellular domain), exon 3 (transmembrane domain) and exon 4 (cytoplasmic domain); and a soluble form (sCTLA4) in which exon 3 is lost (Teft *et al.,* 2006). The soluble form is expressed by resting T cells and its expression disappears within 48 h of activation (Oaks *et al.,* 2000). In activated T cells, the flCTLA4 serves as a transmembrane receptor to inhibit cell proliferation. By contrast, the role of sCTLA4 is not yet known, although it has been shown to act as a functional receptor for B7 antigens, suggesting that sCTLA4 can block the B7-CD28 interaction and interfere with the co-stimulation signal, inhibiting T-cell proliferation (Teft *et al.,* 2006).

Recent studies have shown that the function of regulatory T cells is impaired in autoimmune diseases. The lack of *CTLA4* in knockout mice produces important lymphoproliferative disorders, multi-organ destruction and premature death (Waterhouse *et al.,* 1995). Moreover, *CTLA4* siRNA knock-down in animals leads to the development of autoimmune diabetes (Chen *et al.,* 2006).

In the present study we analyzed the association of two polymorphic sites in the *CTLA4* gene i.e., exon 1: +49A/G and in the 3' UTR region: CT60A/G with vitiligo susceptibility in Gujarat population. We report an association between CT60A/G polymorphism and susceptibility to vitiligo in the Gujarat population. In particular, we found that the presence of CT60GG genotype was more prevalent among vitiligo patients. On contrary, studies from Caucasian and Romanian populations did not find association of CT60A/G polymorphism with vitiligo (LaBerge *et al.,* 2008; Birlea *et al.,* 2009). Another study from Caucasian population showed significant association of the polymorphism only with vitiligo patients having other autoimmune diseases however, this study involved very less sample size (Blomhoff *et al.,* 2005). We did

not find any significant association of genotype and allele frequencies for +49A/G polymorphism in vitiligo patients and controls. In addition, studies from South Indian, Iranian and Romanian populations did not show association of +49A/G polymorphism in vitiligo patients (Deeba *et al.,* 2010; Fattahi *et al.,* 2005; Birlea *et al.,* 2009).

Recently, Birlea et al. (2011) analyzed 104 SNPs in *CTLA4* gene and found one promoter SNP (rs12992492) showing maximum association with both generalized vitiligo (GV) patients and patients having other autoimmune diseases; however patients with isolated GV without other autoimmune diseases did not achieve the study significance threshold for any other SNPs in *CTLA4* gene. This study together with other previous observations suggests that association of *CTLA*4 with GV is secondary, driven by primary genetic association of *CTLA*4 with other autoimmune diseases that are associated with vitiligo. However, the present study did not include vitiligo patients having other autoimmune diseases and surprisingly this study found the significant association of CT60A/G polymorphism with vitiligo patients having no other autoimmune diseases. The deviation of the study from the previous reports may be due to ethnic differences.

Furthermore, to evaluate the possible expression dysregulation of *CTLA*4 isoforms important in T regulatory cell's function, the transcript levels of fl*CTLA*4 and s*CTLA*4 genes were measured in patients with vitiligo and compared with those from unaffected subjects. This is the first report where *CTLA*-4 expression levels were studied in patients with vitiligo. Interestingly, we found decreased mRNA expression of both fl*CTLA*4 and s*CTLA*4 in vitiligo patients as compared to controls. We further analyzed whether the polymorphisms examined in this study influenced the expression levels of fl*CTLA*4 and s*CTLA*4. The CT60A/G dimorphism has been postulated to affect alternative splicing of the *CTLA*4 gene, resulting in decreased levels of soluble CTLA4 (sCTLA4) mRNA which is in concordance with our results (Ueda *et al.,* 2003). Recently, Gerold et al. (2011) has also shown decreased s*CTLA*4 levels in Type-1 diabetic condition and suggested that lower s*CTLA*4 expression may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. In contrast, increased serum sCTLA4 levels were detected in other autoimmune diseases such as Graves' disease (Daroszweski *et al.,* 2009), autoimmune thyroid disease (Saverino 2007), psoriasis (Luszczek *et al.,* 2006) and Crohn's disease (Chen

et al., 2011). These studies suggest that s*CTLA*4 may contribute to the development of autoimmune diseases, probably through inhibiting the B7-flCTLA4 interaction and down-regulation of T cell activation.

In present study the +49A/G genotypes did not influence fl*CTLA*4 and s*CTLA*4 mRNA expression suggesting that it could not contribute major role in vitiligo susceptibility of Gujarat population. Nevertheless, its associations with other diseases are also controversial. Previously, the +49A/G variation was found to affect the cell surface expression of *CTLA*4 but did not affect the expression in other study (Anjos *et al.,* 2002; Munthe-Kaas *et al.,* 2004).

The 3' UTR of *CTLA*4 gene has been found to be involved in several autoimmune diseases, hence to study the genetic variation of such regions is imperative. Interestingly, 3' UTR CT60G allele greatly reduced the mRNA expression of both fl*CTLA*4 and s*CTLA*4 in vitiligo patients as compared to controls suggesting its crucial role in pathogenesis of vitiligo. Further, the haplotype AG (+49A:CT60G) greatly decreased mRNA levels of s*CTLA*4 in patients than fl*CTLA*4 as compared to controls revealing the positive correlation of CT60G in vitiligo pathogenesis.

Moreover, s*CTLA*4/fl*CTLA*4 mRNA ratio was found to be increased with +49AG and CT60GG genotype in vitiligo patients as compared to AA genotypes suggesting G allele to confer lower mRNA levels of s*CTLA*4. Atabani et al. (2005) also found elevated sCTLA4 to flCTLA4 mRNA expression with the CT60 GG and AA genotypes. However, Anjos et al. (2005) and Mayans et al. (2007) could not find effect of the CT60 polymorphism on the expression of s*CTLA*4 and fl*CTLA*4. The present study found higher prevalence of CT60GG genotypes among vitiligo patients however, CT60AA genotype does not seem to modulate *CTLA*4 mRNA expression and hence patients harboring it may have other genetic factors involved in disease pathogenesis supporting the fact that vitiligo may have varied type of precipitating factors. In addition, patients harboring CT60GG genotype had reduced levels of *CTLA*4 mRNA as compared to controls suggestive of CT60GG genotype having a profound effect on *CTLA*4 in progression of disease.

CTLA4 may modulate immune response in two ways: as a cell membrane receptor transmitting a negative signal to the T cell upon ligand binding and as a soluble

molecule that may bind to the same ligand, preventing in this way the positive stimulation of the T cell via CD28 or the negative signaling via cell membrane CTLA4 (Teft *et al.,* 2006; Pawlak *et al.,* 2005). These different interactions may, in part, be responsible for the contradictory results of previous studies of associations of *CTLA*4 polymorphisms with autoimmune diseases. Therefore, further research on *CTLA*4 polymorphisms, dynamics of full length versus s*CTLA*4 expression and turnover in vitiligo as well as, other autoimmune diseases should clarify the role of *CTLA*4 in the regulation of immune response in physiological and pathological situations.

In conclusion, our findings suggest that the dysregulated *CTLA*4 expression in vitiligo patients could result, at least in part, from variations at the genetic level and this study has been published [Dwivedi M, Laddha NC, Imran M, Shah BJ and Begum R (2011). Cytotoxic T-lymphocyte associated antigen-4 (CTLA4) in isolated vitiligo: a genotype-phenotype correlation. Pigment Cell Melanoma Res. 24: 737-740]. For the first time, we show that the 3' UTR CT60A/G polymorphism of the *CTLA*4 gene influences both full length and soluble *CTLA*4 mRNA levels in vitiligo patients, and thus this genotype-phenotype correlation of *CTLA*4 supports the autoimmune pathogenesis of vitiligo.

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