

RESEARCH ARTICLE

A case-control study on association of proteasome subunit beta 8 (*PSMB8*) and transporter associated with antigen processing 1 (*TAP1*) polymorphisms and their transcript levels in vitiligo from Gujarat

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Abstract

Background

Autoimmunity has been implicated in the destruction of melanocytes from vitiligo skin. Major histocompatibility complex (MHC) class-II linked genes proteasome subunit beta 8 (*PSMB8*) and transporter associated with antigen processing 1 (*TAP1*), involved in antigen processing and presentation have been reported to be associated with several autoimmune diseases including vitiligo.

Objectives

To explore *PSMB8* rs2071464 and *TAP1* rs1135216 single nucleotide polymorphisms and to estimate the expression of *PSMB8* and *TAP1* in patients with vitiligo and unaffected controls from Gujarat.

Methods

PSMB8 rs2071464 polymorphism was genotyped using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) and *TAP1* rs1135216 polymorphism was genotyped by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) in 378 patients with vitiligo and 509 controls. Transcript levels of *PSMB8* and *TAP1* were measured in the PBMCs of 91 patients and 96 controls by using qPCR. Protein levels of *PSMB8* were also determined by Western blot analysis.

Results

The frequency of 'TT' genotype of *PSMB8* polymorphism was significantly lowered in patients with generalized and active vitiligo ($p = 0.019$ and $p = 0.005$) as compared to controls suggesting its association with the activity of the disease. However, *TAP1* polymorphism was not associated with vitiligo susceptibility. A significant decrease in expression of

PSMB8 at both transcript level ($p = 0.002$) as well as protein level ($p = 0.0460$) was observed in vitiligo patients as compared to controls. No significant difference was observed between patients and controls for *TAP1* transcripts ($p = 0.553$). Interestingly, individuals with the susceptible CC genotype of *PSMB8* polymorphism showed significantly reduced *PSMB8* transcript level as compared to that of CT and TT genotypes ($p = 0.009$ and $p = 0.003$ respectively).

Conclusions

PSMB8 rs2071464 was associated with generalized and active vitiligo from Gujarat whereas *TAP1* rs1135216 showed no association. The down-regulation of *PSMB8* in patients with risk genotype 'CC' advocates the vital role of *PSMB8* in the autoimmune basis of vitiligo.

Introduction

Vitiligo, a cosmetic disfigurement disorder, may lead to psychological and social stigma, particularly in people with dark and intermediate skin tones. It is characterized by circumscribed milky white patches on the skin affecting about 0.06–2.28% of the world population [1]. Based on a few dermatological outpatient records, the prevalence of vitiligo is found to be 0.5 to 2.5% in India [2], wherein Gujarat and Rajasthan states have the high prevalence i.e. ~8.8% [3]. The exact etiopathology of vitiligo is not defined, however, based on extensive studies various theories such as oxidative stress, autoimmunity, and neurochemical hypothesis have been proposed to explain the underlying pathomechanisms [4–7]. Autoimmunity has been strongly involved in the development of disease, as 30% of vitiligo cases are affected with at least one of the concomitant autoimmune disorders [5,6]. Several studies including ours have identified critical role of CD8⁺ cytotoxic T cells in melanocyte destruction [8,9]. Generation of antigenic peptides and their transport across the membrane of the endoplasmic reticulum for assembly with major histocompatibility complex (MHC) class I molecules are essential steps in antigen presentation to cytotoxic T lymphocytes [10]. Genes within MHC class II loci along with genes involved in antigen processing and presentation i.e., proteasome subunit beta 8 (*PSMB8*) and transporter associated with antigen processing 1 (*TAP1*) have been reported to be associated with several autoimmune diseases including vitiligo [11–19]. The *PSMB8*, often referred as *LMP7* encodes interferon (IFN)- γ inducible subunit of immune proteasome i.e., $\beta 5i$ involved in degradation of ubiquitinated intracellular proteins into peptides that are especially suited for presentation by MHC class I molecules. Whereas, *TAP1* encode a subunit of an IFN- γ inducible heterodimer which binds with peptides cleaved by the proteasome and transports them to be loaded into nascent MHC class I molecules for presentation to CD8⁺ T cells [20,21].

The genome-wide association study (GWAS) on generalized vitiligo revealed that the association of *TAP1-PSMB8* seems to derive from linkage disequilibrium with major primary signals in the MHC class I and class II regions [17]. Out of 8 different single nucleotide polymorphisms (SNPs) of *PSMB* and *TAP* gene region studied, *PSMB8* intron 6 G/T and *TAP1* exon 10 A/G were found to be significantly associated with vitiligo in the Western population [14]. Another study showed significant association of *TAP1* exon 10 A/G polymorphism with vitiligo in Saudi population but not for *PSMB8* intron 6 G/T polymorphism [22]. The nature of the genetic association may vary according to different ethnic backgrounds. However,

despite having high prevalence of vitiligo in Gujarat, there are no reports of *PSMB8* and *TAP1* polymorphisms so far. Hence, the present study aims, (i) to investigate the association of *PSMB8* intron 6 (rs2071464) and *TAP1* exon 10 (rs1135216) polymorphisms and (ii) to estimate transcript levels of *PSMB8* and *TAP1* using a case-control approach.

Materials and methods

Study subjects

We report a case-control study including 509 ethnically age and gender matched controls and 378 patients with vitiligo from Gujarat. Unaffected individuals of age between 5 to 60 years were recruited in the study. None of the unaffected individuals had any evidence of vitiligo and any other disease. Patients with vitiligo who referred to S.S.G. Hospital at Vadodara, Gujarat, India were recruited in the study. The inclusion criteria followed were: outpatients of age between 5 to 60 years and both the parents should be Gujarati by birth. Patients with other diseases and those unwilling to participate in the study were excluded. The diagnosis of vitiligo by dermatologists was clinically based on characteristic skin depigmentation with typical localization and white color lesions on the skin, under Woods lamp. Generalized or non-segmental vitiligo (GV) was characterized by depigmented patches varying in size from a few to several centimeters in diameter, involving one or both sides of the body with a tendency towards symmetrical distribution [23]. Whereas localized or segmental vitiligo (LV) typically has a rapidly progressive but limited course, depigmentation spreads within the segment during a period of 6–24 months and then stops; further extension is rare [23]. Following clinical criteria proposed by Falabella *et al.*, [24] and discussed in the Vitiligo Global Issues Consensus Conference 2012 [23], were used for characterizing stable vitiligo (SV): (i) lack of progression of old lesions within the past 2 years; (ii) no new lesions developing within the same period. Active vitiligo (AV) was defined as the appearance of new lesions and spreading of existing lesions observed during past two-year duration. The importance of the study was explained to all participants and written consent was obtained. Informed consent in written was obtained from the next of kin, caretakers, or guardians on behalf of the minors/children enrolled in the study. The study plan and consent forms were approved by the Institutional ethical committee for human research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/BC/RB/1). Demographic characteristics of the patients are provided in Supporting information as ‘S1 Table’.

Genomic DNA extraction

Genomic DNA was extracted from PBMCs using ‘QIAampTM DNA Blood Kit’ (QIAGEN Inc., Valencia, CA 91355, USA) according to the manufacturer’s instructions. After extraction, concentration and purity of DNA were estimated spectrophotometrically, quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20°C until further analyses.

Genotyping of *PSMB8* rs2071464 polymorphism

Polymerase chain reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) technique was used to genotype *PSMB8* rs2071464 polymorphism. The primers used for polymerase chain reaction are mentioned in S2 Table. The reaction mixture of the total volume of 20 µL included 3 µL (100ng) of genomic DNA, 11 µL nuclease-free H₂O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (GeneiTM, Bangalore, India), 1 µL of 10 pM corresponding forward and reverse primers (EurofinsTM, India), and 0.3 µL (3 U/µL) Taq Polymerase (GeneiTM,

Bangalore, India). Amplification was performed Eppendorf Mastercycler Gradient Thermocycler (EppendorfTM, Germany) according to the protocol: 95°C for 10 minutes followed by 45 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds, and 72°C for 10 minutes. The amplified products were checked by electrophoresis on a 2.0% agarose gel stained with ethidium bromide. Restriction enzyme was used for digesting the PCR product (S2 Table). 15 µL of the amplified products were digested with 1U of *Hha* I (FermentasTM, Thermo Scientific, Waltham, MA) in a total reaction volume of 20µL as per the manufacturer's instruction. The digestion products were resolved with 50 bp DNA ladder (NovagenTM, Perfect DNA ladder) on 3.5% agarose gel stained with ethidium bromide and visualized under E-Gel Imager (Life TechnologiesTM, Carlsbad, CA). Representative gel image is shown in S1 Fig. 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). Six samples of each genotype were also confirmed by sequencing (S2 Fig) using carefully designed primers (S3 Table).

Genotyping of *TAP1* rs1135216 polymorphism

TAP1 rs1135216 polymorphism was genotyped using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method. DNA was amplified in two different PCR reactions with a generic antisense primer and one of the two allele-specific sense primers (S2 Table). To assess the success of PCR amplification in both the reactions, an internal control of 407 bp was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone (*HGH*) (S2 Table). The reaction mixture of the total volume of 15 µL included 3 µL (100 ng) of genomic DNA, 4.7 µL nuclease-free H₂O, 1.5 µL 10x PCR buffer, 1.5 µL 2mM dNTPs (GeneiTM, Bangalore, India), 1 µL of 10 pM allele-specific and common primers (EurofinsTM, India), 1 µL of 10 pM control primers (*HGH*), and 0.3 µL (3U/µL) Taq Polymerase (GeneiTM, Bangalore, India). Amplification was performed using a Mastercycler Gradient PCR (EppendorfTM, Germany) according to the protocol: 95°C for 10 minutes followed by 45 cycles of 95°C for 30 seconds, 61°C for 30 seconds, and 72°C for 30 seconds, and 72°C for 10 min. The PCR products were resolved on 3.5% agarose gel stained with ethidium bromide along with 50bp DNA ladder (NovagenTM, Perfect DNA ladder) and visualized under E-Gel Imager (Life TechnologiesTM, Carlsbad, CA). Two amplicons were available for each sample (one each specific for A or G allele). Representative gel image is shown in S1 Fig. 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). Six samples of each genotype were also confirmed by sequencing (S3 Fig) using carefully designed primers (S3 Table).

Estimation of *PSMB8* and *TAP1* transcript levels

RNA extraction and cDNA synthesis. Total RNA from PBMCs was isolated and purified using the Ribopure-blood Kit (AmbionTM Inc., Austin, TX, U.S.A.) 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 (AmbionTM inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. cDNA synthesis was performed using 1 µg of total RNA by RevertAid First Strand cDNA Synthesis Kit (FermentasTM, Vilnius, Lithuania) according to the manufacturer's instructions in Eppendorf Mastercycler Gradient Thermocycler (EppendorfTM, Germany).

Quantitative realtime PCR (qPCR). The expression of *PSMB8*, *TAP1* and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) transcripts were measured by qPCR using gene

specific primers (EurofinsTM, Bangalore, India) as shown in [S4 Table](#). Expression of the *GAPDH* gene was used as a reference. qPCR was performed in duplicates in 20 μ l volume using LightCycler[®] 480 SYBR Green I Master (RocheTM Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing, and extension (95°C for 10 sec, 65°C for 15 sec, 72°C for 20 sec). The fluorescence data collection was performed during the extension step. At the end of the amplification phase, a melt curve analysis was carried out to check the specificity of the products formed. The PCR cycle at which PCR amplification begins its exponential phase and product fluorescence intensity finally rises above the background and becomes visible was considered as the crossing point-PCR-cycle (C_p) or cycle threshold (C_T). The ΔC_p value was determined as the difference between the cycle threshold of target genes (*PSMB8/TAP1*) and reference gene (*GAPDH*). The difference between the two ΔC_p values (ΔC_p Controls and ΔC_p patients) was considered as $\Delta\Delta C_p$ to obtain the value of fold expression ($2^{-\Delta\Delta C_p}$).

Estimation of PSMB8 protein expression

Western blot analysis. Five ml blood was drawn from healthy controls and patients with active GV and collected in EDTA vials. Red blood cells were lysed with RBC lysis buffer (0.17 M Tris/ 0.16 M NH_4Cl pH 7.2) and the remaining leukocytes were washed in PBS, and lysed in lysis buffer (1 mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) containing 1x proteinase inhibitors (Sigma, Bangalore, India). Protein concentration was determined by Bradford assay (HiMedia Laboratories, India) and 20 μ g protein was loaded on 12% SDS-PAGE along with Precision Plus Protein[™] Dual Color Standards (Bio-Rad, Germany). Protein was electro-blotted on PVDF membrane at 100 V for 1.5 hrs. Following the transfer, the membrane was blocked with 5% blocking buffer (5% BSA and 0.1% Tween-20 in PBS) for 1 hr at room temperature. The membrane was incubated overnight with primary antibody against LMP7/PSMB8 (ab58094). After incubation the membrane was washed four times with PBS-T (PBS containing 0.1% Tween 20) for 15 min. and incubated with a secondary anti-mouse antibody (Bangalore Genei, India) at room temperature for 1 hr. The membrane was similarly washed four times with PBS-T and protein bands on the membrane were then visualized by using Bio-Rad Clarity[™] western ECL substrate (Bio-Rad, Germany) and signal was scanned using the Chemidoc[™] Touch Gel Imaging System (Bio-Rad, Germany). Intensities of target proteins were normalized with that of total protein loading by staining the membrane with Ponceau. Densitometric analysis of the protein bands was calculated by ImageJ software.

Statistical analyses

Hardy-Weinberg equilibrium (HWE) was evaluated for both SNPs in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. Distribution of the genotypes and allele frequencies of polymorphisms in different groups were compared using chi-square test with 2x2 contingency tables. Major genotype/allele was used as a reference. Multiple comparisons were controlled by the Bonferroni's method. Odds ratio (OR) with 95% confidence interval (CI) for disease susceptibility was also calculated. Haplotype and LD analysis were carried out using <http://analysis.bio-x.cn/myAnalysis.php> [25]. For analyses of the transcript and protein levels unpaired t-test and one-way ANOVA were applied. Tukey's multiple correction was applied for multiple testing and the p-values were adjusted. All the statistical tests were carried out using Prism 6 software (Graph Pad Software, USA).

Bioinformatics analysis

In silico prediction tools HaploReg v4.1 [26] and Regulome DB [27] were employed to predict the functional impact of non-coding polymorphism. *In silico* prediction tools SIFT [28], PANTHER [29], I-MUTANT SUITE [30], POLYPHEN [31], MUPRO [32] were employed to predict the impact on the protein due to single amino acid variation. SNPs and GO [33] predicts the variation effect which might terminate into a disease like a trait. The details have been described in 'Supporting Information' file (S1 Text).

Results

PSMB8 rs2071464 polymorphism in vitiligo

Genotyping of *PSMB8* intron 6 rs2071464 SNP by PCR-RFLP using *Hha* I and subsequent sequencing results revealed that there is C>T nucleotide change instead of previously reported G>T change, which falls in the *Hha* I recognition/restriction site and was imputed to *PSMB8* rs2071464 SNP [12,19,34,35]. The observed genotype frequencies of *PSMB8* rs2071464 SNP among the controls were in accordance ($p = 0.071$) whereas, genotype frequencies among the patients were deviated ($p = 0.001$) from HWE. When 'C' allele and CC genotype were used as reference group, the frequencies of the variant 'T' allele and homozygous 'TT' genotype were significantly lower in patients with vitiligo as compared to controls (49% vs. 54%, $p = 0.031$; 19% vs. 27%, $p = 0.026$ respectively) but it did not remain significant after Bonferroni's correction. The protective role of 'TT' genotype in patients was suggested by OR = 0.629 (95% CI = 0.41–0.94). OR suggests that the minor allele 'T' might have the protective role in the disease pathogenesis (Table 1). Analysis based on types of vitiligo revealed significantly lower frequency of 'TT' genotype (18% vs. 27%, $p = 0.019$) and 'T' allele (48% vs. 54%, $p = 0.024$) in patients with GV as compared to controls. No significant difference in genotype and allele frequencies between patients with LV in comparison to patients with GV or controls (Table 2). Interestingly, a similar trend was observed upon analysis based on the activity of the disease (Table 3). Predominantly increased frequency of the risk genotype 'CC' (24% vs. 19%) and allele 'C' (53% vs. 46%) was observed in patients with AV as compared to controls. The frequency of the protective genotype 'TT' (18% vs. 27%, $p = 0.005$) and allele 'T' (47% vs. 54%, $p = 0.007$) was significantly lowered in comparison to controls. However, no significant difference in allele and genotype frequencies was observed between patients with AV and SV.

TAP1 rs1135216 polymorphism in Vitiligo

Both, control and patient groups were following HWE ($p = 0.663$ and $p = 0.167$ respectively; Table 1). Major allele 'A' and 'AA' genotype were considered as the reference. The allele and genotype frequencies were not significantly different in patients and control (Table 1). *TAP1* SNP when analyzed based on the type of vitiligo, no significant difference in genotype and allele frequencies was observed between patients with GV and LV with respect to unaffected controls (Table 2). Analysis based on the activity of the disease also showed no significant difference among the genotypes as well as allele frequencies (Table 3).

Linkage disequilibrium and haplotype analyses

LD analysis revealed that two polymorphisms investigated i.e., *PSMB8* rs2071464 and *TAP1* rs1135216 were in low LD association ($D' = 0.432$, $r^2 = 0.044$). Haplotype evaluation of the two polymorphic sites was performed and the estimated frequencies of the haplotypes were not significantly different between patients and controls (global $p = 0.278$; Table 4).

Table 1. Association of *PSMB8* and *TAP1* polymorphisms in patients with vitiligo from Gujarat.

SNP	Genotype/Allele	Patients n = 378 (Freq.)	Controls n = 509 (Freq.)	p for Association	Odds ratio	CI (95%)	p for HWE
<i>PSMB8</i> rs2071464	Genotype						0.071 (C) 0.001 (P)
	CC	82 (0.22)	97 (0.19)	R	1	-	
	CT	222 (0.59)	273 (0.54)	0.825 ^a	0.961 ^a	0.68–1.35 ^a	
	TT	74 (0.19)	139 (0.27)	0.026 ^a	0.629 ^a	0.41–0.94 ^a	
	Allele						
	C	386 (0.51)	467 (0.46)	R	1	-	
T	370 (0.49)	551 (0.54)	0.031 ^b	0.812 ^b	0.67–0.98 ^b		
<i>TAP1</i> rs1135216	Genotype						0.663 (C) 0.167 (P)
	AA	263 (0.70)	341 (0.67)	R	1	-	
	AG	100 (0.26)	153 (0.30)	0.278 ^a	0.847 ^a	0.63–1.14 ^a	
	GG	15 (0.04)	15 (0.04)	0.487 ^a	1.297 ^a	0.62–2.70 ^a	
	Allele						
	A	626 (0.83)	835 (0.82)	R	1	-	
G	130 (0.17)	183 (0.18)	0.670 ^b	0.950 ^b	0.74–1.21 ^b		

'n' represents number of Patients/ Controls,

'R' represents reference group,

HWE refers to Hardy-Weinberg Equilibrium,

CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.

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

^aPatients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table,

^bPatients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table,

Statistical significance was considered at p value ≤ 0.025 due to Bonferroni's correction.

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PSMB8 transcript and protein levels in vitiligo

Analysis of *PSMB8* transcript levels revealed a significant decrease in expression of *PSMB8* transcripts in patients as compared to controls ($p = 0.002$; Fig 1A) after normalization with *GAPDH* expression. The $2^{-\Delta\Delta C_p}$ analysis showed approximately 0.52-fold decrease in the expression of *PSMB8* transcript levels in patients, as compared to controls (Fig 1A). Interestingly, analysis based on type and activity of the disease revealed that *PSMB8* transcript levels were significantly decreased in patients with GV as well as AV in comparison to controls ($p = 0.007$ and $p = 0.006$ respectively; Fig 1B and 1C), suggesting a role in the autoimmune basis of the disease. However, there was no significant difference in patients with LV and SV as compared to controls ($p = 0.090$ and $p = 0.112$ respectively; Fig 1B and 1C). Also, no significant difference in transcript levels was observed between GV vs LV and AV vs SV patients (Fig 1B and 1C). When expression of *PSMB8* transcripts was monitored in different age at onset groups of patients, no significant difference was observed in any of the age of onset groups i.e., 21–40, 41–60 and 61–80 years when compared with 1–20 years (Fig 1D). Gender-based analysis also showed no significant difference in *PSMB8* transcripts in both the groups ($p = 0.396$; Fig 1E).

Furthermore, the decreased transcript expression of *PSMB8* in patients with vitiligo was confirmed at protein level by western blot analysis in PBMCs of healthy controls (n = 6) and

Table 2. Association of *PSMB8* and *TAP1* polymorphisms in patients with generalized and localized vitiligo from Gujarat.

SNP	Genotype / Allele	Generalized Vitiligo n = 292 (Freq.)	Localized Vitiligo n = 86 (Freq.)	Controls n = 509 (Freq.)	p for Association	Odds ratio	CI (95%)
<i>PSMB8</i> rs2071464	Genotype						
	CC	64 (0.22)	18 (0.21)	97 (0.19)	R	1	-
	CT	174 (0.60)	48 (0.56)	273 (0.54)	0.951 ^a	1.020 ^a	0.55–1.88 ^a
					0.854 ^b	0.966 ^b	0.67–1.40 ^b
					0.858 ^c	0.947 ^c	0.52–1.70 ^c
	TT	54 (0.18)	20 (0.23)	139 (0.27)	0.461 ^a	0.759 ^a	0.36–1.58 ^a
					0.019 ^b	0.588 ^b	0.37–0.92 ^b
					0.468 ^c	0.775 ^c	0.39–1.54 ^c
	Allele						
	C	302 (0.52)	84 (0.49)	467 (0.46)	R	1	-
T	282 (0.48)	88 (0.51)	551 (0.54)	0.507 ^a	0.891 ^a	0.63–1.25 ^a	
				0.024 ^b	0.791 ^b	0.64–0.97 ^b	
				0.471 ^c	0.887 ^c	0.64–1.23 ^c	
<i>TAP1</i> rs1135216	Genotype						
	AA	203 (0.69)	60 (0.70)	341 (0.67)	R	1	-
	AG	78 (0.27)	22 (0.26)	153 (0.30)	0.868 ^a	1.048 ^a	0.60–1.82 ^a
					0.347 ^b	0.856 ^b	0.62–1.18 ^b
					0.450 ^c	0.817 ^c	0.48–1.38 ^c
	GG	11 (0.04)	4 (0.04)	15 (0.04)	0.730 ^a	0.812 ^a	0.25–2.64 ^a
					0.608 ^b	1.232 ^b	0.55–2.73 ^b
					0.470 ^c	1.516 ^c	0.49–4.72 ^c
	Allele						
	A	484 (0.83)	142 (0.88)	835 (0.82)	R	1	-
G	100 (0.17)	30 (0.12)	183 (0.18)	0.922 ^a	0.978 ^a	0.62–1.53 ^a	
				0.666 ^b	0.942 ^b	0.72–1.23 ^b	
				0.866 ^c	0.964 ^c	0.63–1.47 ^c	

'n' represents number of Patients/ Controls,

'R' represents reference group,

CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.

^aGeneralized vitiligo vs. Localized vitiligo,

^bGeneralized vitiligo vs. Controls,

^cLocalized vitiligo vs. Controls,

Statistical significance was considered at $p < 0.025$ due to Bonferroni's correction.

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Table 3. Association of *PSMB8* and *TAP1* polymorphisms in patients with active and stable vitiligo from Gujarat.

SNP	Genotype / Allele	Active Vitiligo n = 305 (Freq.)	Stable Vitiligo n = 73 (Freq.)	Controls n = 509 (Freq.)	p for Association	Odds ratio	CI (95%)	
<i>PSMB8</i> rs2071464	Genotype							
	CC	72 (0.24)	10 (0.16)	97 (0.19)	R	1	-	
	CT	178 (0.58)	44 (0.60)	273 (0.54)	0.123 ^a	0.562 ^a	0.27–1.18 ^a	
					0.478 ^b	0.878 ^b	0.61–1.26 ^b	
					0.224 ^c	1.563 ^c	0.76–3.23 ^c	
	TT	55 (0.18)	19 (0.24)	139 (0.27)	0.031 ^a	0.402 ^a	0.17–0.93 ^a	
					0.005 ^b	0.533 ^b	0.34–0.82 ^a	
					0.493 ^c	1.326 ^c	0.59–2.98 ^c	
	Allele							
	C	322 (0.53)	64 (0.44)	467 (0.46)	R	1	-	
T	288 (0.47)	82 (0.56)	551 (0.54)	0.052 ^a	0.698 ^a	0.48–1.00 ^a		
				0.007 ^b	0.758 ^b	0.62–0.93 ^b		
				0.644 ^c	1.086 ^c	0.76–1.54 ^c		
<i>TAP1</i> rs1135216	Genotype							
	AA	205 (0.67)	58 (0.80)	341 (0.67)	R	1	-	
	AG	86 (0.28)	14 (0.19)	153 (0.30)	0.086 ^a	1.738 ^a	0.92–3.28 ^a	
					0.677 ^b	0.935 ^b	0.68–1.28 ^b	
					0.045 ^c	0.538 ^c	0.29–0.99 ^c	
	GG	14 (0.05)	01 (0.01)	15 (0.04)	0.156 ^a	3.961 ^a	0.51–30.77 ^a	
					0.246 ^b	1.553 ^b	0.73–3.28 ^b	
					0.352 ^c	0.392 ^c	0.05–3.02 ^c	
	Allele							
	A	496 (0.81)	130 (0.89)	835 (0.82)	R	1	-	
G	114 (0.19)	16 (0.11)	183 (0.18)	0.026 ^a	1.867 ^a	1.07–3.26 ^a		
				0.719 ^b	1.049 ^b	0.81–1.36 ^b		
				0.035 ^c	0.561 ^c	0.32–0.96 ^c		

'n' represents number of Patients/ Controls,

'R' represents reference group,

CI refers to Confidence Interval, Odds ratio is based on allele frequency distribution.

^aActive Vitiligo vs. Stable Vitiligo,

^bActive Vitiligo vs. Controls,

^cStable Vitiligo vs. Controls,

Statistical significance was considered at $p < 0.025$ due to Bonferroni's correction.

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Table 4. Distribution of haplotypes frequencies for *PSMB8* (C/T) and *TAP1* (A/G) polymorphisms in vitiligo patients and controls.

Haplotype [<i>PSMB8</i> (C/T): <i>TAP1</i> (A/G)]	Patients (Freq) n = 742	Control (Freq) n = 974	p for association	p (Global)	Odds Ratio [95%CI]
C A	222 (0.38)	194 (0.31)	0.058	0.278	1.26 [0.99~1.60]
C G	72 (0.12)	75 (0.27)	0.904		0.98 [0.69~1.38]
T A	262 (0.45)	296 (0.15)	0.092		0.82 [0.65~1.03]
T G	30 (0.05)	31 (0.27)	0.908		0.97 [0.58~1.62]

CI represents Confidence Interval,
(Frequency <0.03 in both control & case has been dropped and was ignored in analysis).

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patients with active GV (n = 7). A significant decrease ($p = 0.0460$) in expression of *PSMB8* was observed in patients as compared to controls (Fig 2).

Genotype—phenotype correlation for *PSMB8* rs2071464 polymorphism

Further, the expression of *PSMB8* transcripts was analyzed with respect to *PSMB8* rs2071464 genotypes. Interestingly, *PSMB8* transcript levels were significantly reduced in individuals with the susceptible CC genotype when compared with CT and TT genotypes ($p = 0.009$ and $p = 0.003$, respectively; Fig 1F). However, no significant difference in *PSMB8* transcripts levels was observed between individuals with the CT and TT genotypes (Fig 1F).

TAP1 transcript levels in vitiligo

Analysis of *TAP1* transcript levels was carried out after normalization with *GAPDH* expression. No significant difference in expression of *TAP1* transcripts was observed ($p = 0.553$) between patients and controls (Fig 3A). The $2^{-\Delta\Delta C_p}$ analysis showed approximately 1.12- fold change in expression of *TAP1* transcript in patients as compared to controls (Fig 3A). Analysis based on type of the disease suggested no significant difference in *TAP1* transcript levels in patients with GV and LV in comparison to controls ($p = 0.090$ and $p = 0.219$ respectively; Fig 3B). Moreover, there was no significant difference in patients with AV and SV as compared to controls ($p = 0.671$ and $p = 0.291$ respectively; Fig 3C). When expression of *TAP1* transcripts was monitored in different age at onset groups of patients, no significant difference was observed in any of the age of onset groups i.e., 21–40, 41–60 and 61–80 years when compared with 1–20 years (Fig 3D). Gender-based analysis showed no significant difference in *TAP1* transcripts in both the groups (Fig 3F).

Bioinformatics analyses

Analysis of functional consequences of *PSMB8* rs2071464 by RegulomeDB was scored 6 and classified as having minimal binding evidence (Table 5). HaploReg v4.1 predicted *PSMB8* rs2071464 could alter 7 DNA motifs. RegulomeDB revealed that the Chromatin state is altered favoring strong transcription and genic enhancer by the polymorphism in peripheral blood cells (<http://www.regulomedb.org/snp/chr6/32809075>). Analysis by HaploReg v4.1 further confirmed the enhancer chromatin state in peripheral blood and T cells due to the polymorphism (http://archive.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs2071464).

TAP1 exon 10 A>G leads to variation in *TAP1* protein from Asp to Gly at position 637 [36]. PANTHER tool showed variation Asp to Gly at position 637 is not deleterious for *TAP1* function, with the score of 0.3456 (Table 5). POLYPHEN tool showed that the substitution does not affect the phenotype or have damaging effects on the function of *TAP1* protein.

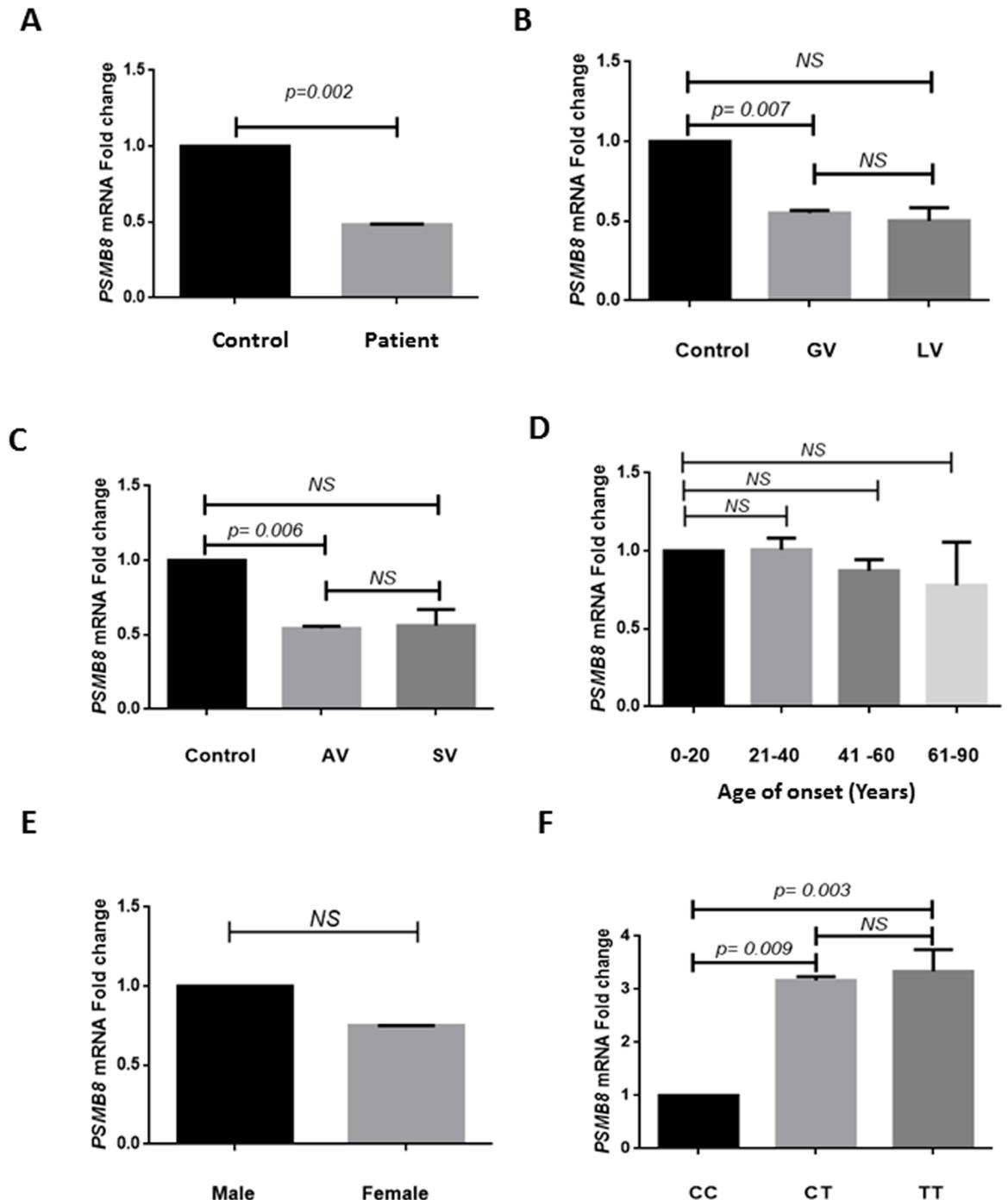


Fig 1. Relative gene expression of *PSMB8* in cases and controls. (A) Expression of *PSMB8* transcripts in 96 controls (52 male and 44 female), 91 patients with vitiligo (48 male and 43 female) was analyzed by applying unpaired t-test. Patients showed a significant decrease in transcript levels of *PSMB8* compared to controls (mean $\Delta\text{Cp} \pm \text{SEM}$: 8.958 ± 0.239 vs 10.01 ± 0.229 ; $p = 0.002$). Expression of *PSMB8* transcripts in patients against controls showed 0.52-fold decrease as determined by the $2^{-\Delta\Delta\text{Cp}}$ method. (B) Expression of *PSMB8* transcripts in 96 controls and 72 patients with GV and 19 patients with LV was analyzed by using one-way ANOVA. Patients with GV showed significantly decreased *PSMB8* transcript levels as compared to controls ($p = 0.007$). However, there was no significant difference in *PSMB8* transcript levels between patients with GV and LV as well as in patients with LV as compared to controls ($p = 0.975$ and $p = 0.090$, respectively). (C) Expression of *PSMB8* transcripts in 96 controls and 69 patients with AV and 22 patients with SV was analyzed by using one-way ANOVA. Patients with AV showed significantly decreased *PSMB8* transcript levels as compared to controls ($p = 0.006$). However, there was no significant difference in *PSMB8* transcript levels between patients with AV and SV as well as in patients with SV as

compared to controls ($p = 0.999$ and $p = 0.112$, respectively). (D) Expression of *PSMB8* transcripts with respect to different age of onset groups in 91 patients with vitiligo was analyzed by using one-way ANOVA. No significant difference in *PSMB8* transcript levels was observed in patients with respect to different age of onset groups. (E) Expression of *PSMB8* transcripts with respect to sex differences in 48 male and 43 female patients was analyzed by applying unpaired t-test. No significant difference was observed in both the groups ($p = 0.396$). (F) Expression of *PSMB8* transcripts with respect to the *PSMB8* rs2071464 SNP in 96 controls and 91 patients was analyzed by using one-way ANOVA. Individuals with the CC genotype showed decreased *PSMB8* transcripts when compared with CT and TT genotypes ($p = 0.009$ and $p = 0.003$, respectively). No significant difference in *PSMB8* transcripts levels was observed in individuals with the CT and TT genotypes ($p = 0.448$).

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I-MUTANT and MUPRO predictions revealed decreased stability of Asp637Gly variants compared to native structure, which might affect the protein function. SNPs AND GO tool revealed that the variant doesn't show disease like trait. (Table 6).

Discussion

The association of MHC region has been implicated in several GWAS on vitiligo including in Indian subcontinent [16,17,19,37–42]. Association of MHC class II region with generalized vitiligo was reported in European-derived white population by Jin *et al.*, [42]. The strong link between autoimmune diseases and MHC class II genes suggests that abnormalities in MHC class II gene products may play a crucial role in vitiligo susceptibility. Interestingly, the association of GV with SNPs in the *PSMB8-TAP1* region of the MHC has been reported to derive from LD with primary association signals in the MHC class I and class II regions [17]. Any alterations in function or expression of *PSMB8* or *TAP1* proteins could potentially affect the antigenic repertoire expressed on the cell surface and may alter peripheral tolerance [43]. Several studies have addressed the association of *PSMB8* and *TAP1* polymorphisms in patients with vitiligo (Table 7); however, studies revealing the impact of these polymorphisms at transcript and protein levels are few.

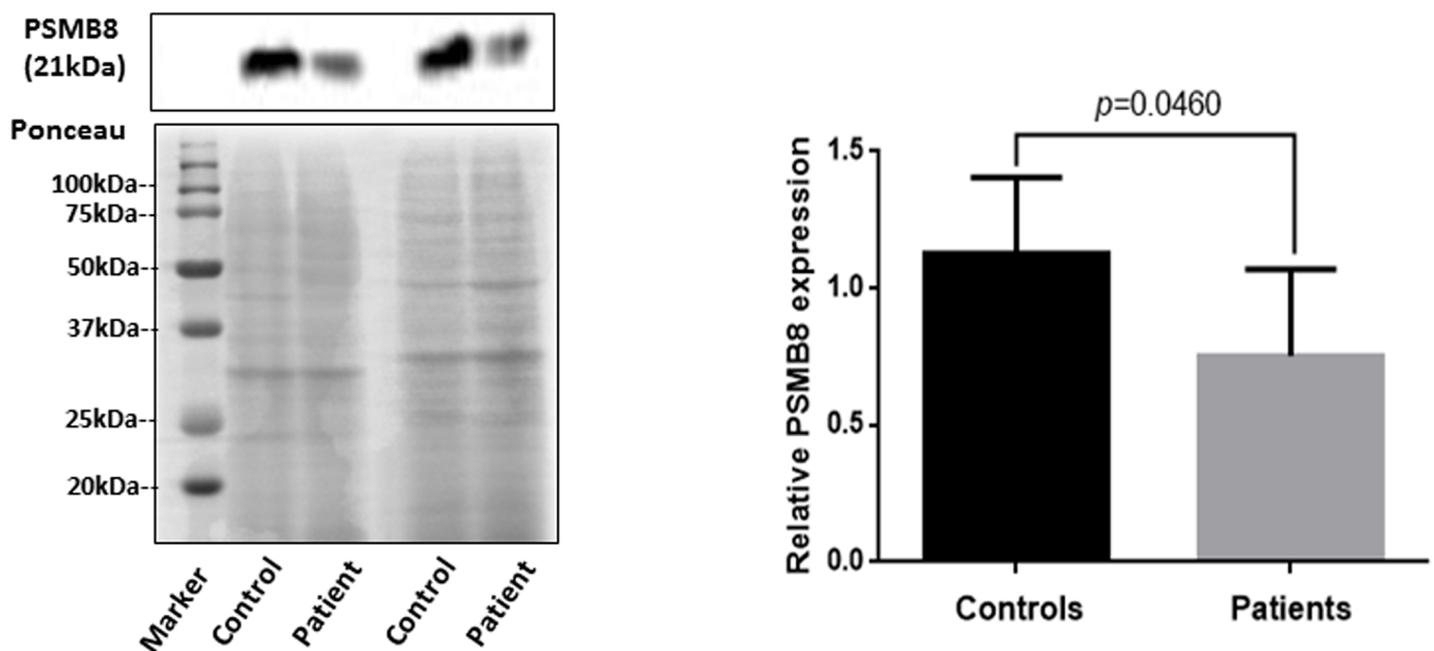


Fig 2. Analysis of *PSMB8* protein expression. Western blot analysis in PBMCs of healthy controls ($n = 6$) and patients with active GV ($n = 7$) revealed significant decrease ($p = 0.0460$) in expression of *PSMB8* after normalization with Ponceau staining.

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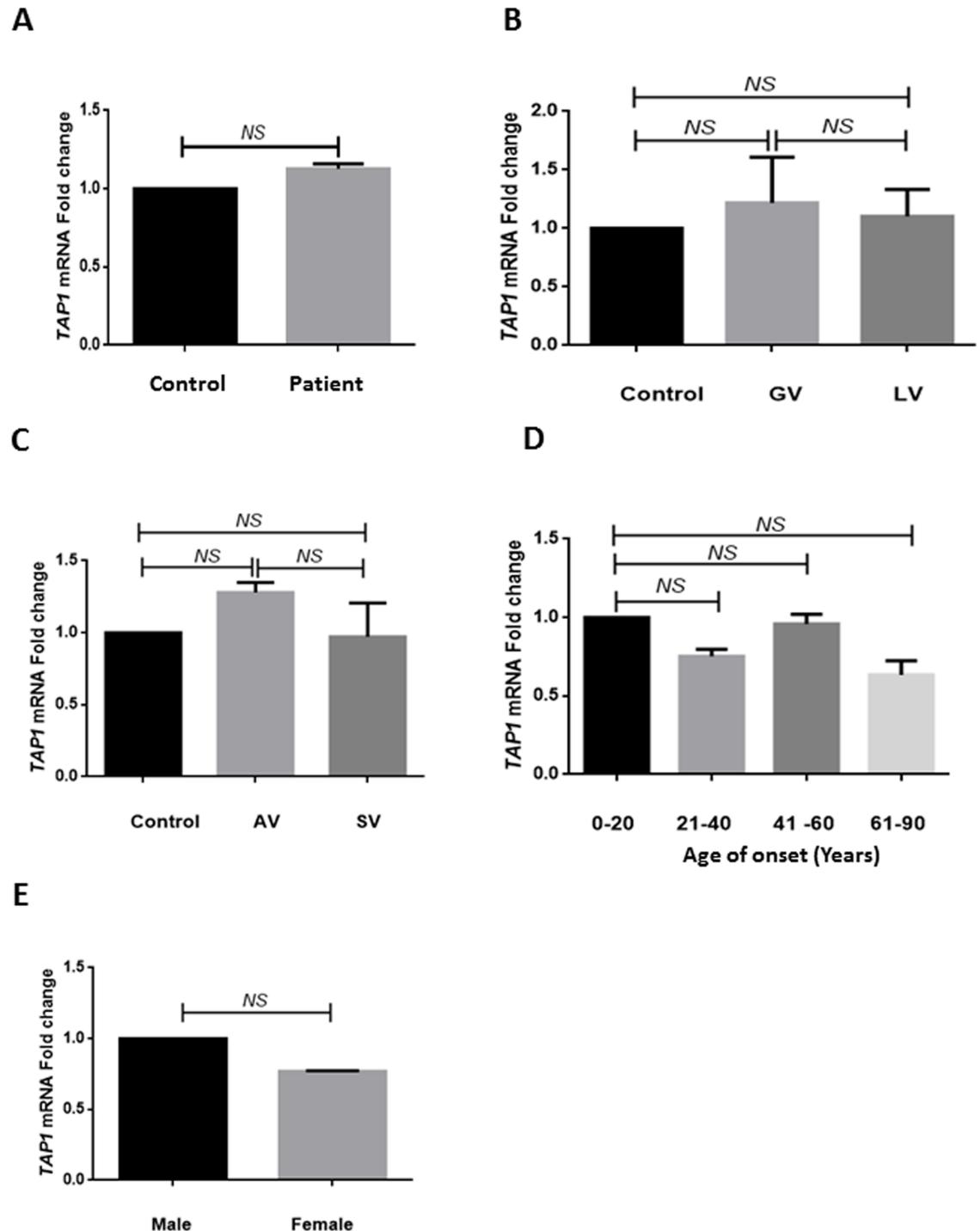


Fig 3. Relative gene expression of *TAP1* in patients and controls. (A) Expression of *TAP1* transcripts in 96 controls, 91 patients with vitiligo was analyzed by applying unpaired t-test. No significant difference in transcript levels of *TAP1* was observed as compared to controls (mean $\Delta\text{Cp} \pm \text{SEM}$ 5.59 \pm 0.188 vs 5.421 \pm 0.228; $p = 0.553$). Expression of *TAP1* transcripts in controls and patients with vitiligo showed approximately 1.12-fold change (NS) as determined by the $2^{-\Delta\Delta\text{Cp}}$ method. (B) Expression of *TAP1* transcripts in 96 controls and 72 patients with GV and 19 patients with LV was analyzed by using one-way ANOVA. Patients with GV and LV showed no significant difference in *TAP1* transcript levels as compared with controls ($p = 0.856$ and $p = 0.090$, respectively). No significant difference in *TAP1* transcript levels was observed between GV and LV ($p = 0.219$). (C) Expression of *TAP1* transcripts in 96 controls and 69 patients with AV and 22 patients with SV was analyzed by using one-way ANOVA. Patients with AV and SV showed no significant difference in *TAP1*

transcripts levels as compared with controls ($p = 0.671$ and $p = 0.291$, respectively). No significant difference in *TAP1* transcript levels was observed among patients with AV and SV ($p = 0.634$). (D) Expression of *TAP1* transcripts with respect to different age of onset groups in 91 patients with vitiligo was analyzed by using one-way ANOVA. No significant difference in *TAP1* transcripts levels was observed in patients with respect to different age of onset groups. (F) Expression of *TAP1* transcripts with respect to sex differences in 48 male patients and 43 female patients was analyzed by applying unpaired t-test. No significant difference was observed in both the groups ($p = 0.444$).

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The present study suggests the association of *PSMB8* rs2071464 SNP with GV as well as with the disease activity (AV); however, *TAP1* rs1135216 SNP was not associated with vitiligo in Gujarat. Our results are in accordance with the previous study [14] reported in Western population for *PSMB8* SNP. In contrast, two studies have found *TAP1* exon 10 SNP to be associated with vitiligo in Saudi population, and this may be due to differences in the ethnicity [22,45]. Birlea *et al.*, [18] have addressed 34 SNPs spanning *TAP1-PSMB8* region in GWAS and the meta-analysis study in GV patients; however, no association was observed for *TAP1* rs1135216 and *PSMB8* rs2071627 SNPs.

The *PSMB8* encodes IFN- γ inducible subunit (b5i/LMP7) of the immunoproteasome, which degrades the ubiquitin-tagged cytoplasmic proteins into peptides that are especially suited for presentation by MHC class I molecules to CD8⁺ cytotoxic T cells [46]. Significant association of *PSMB8* rs2071464 leads us to speculate some functional consequences of this SNP in the disease pathogenesis. Intriguingly, the decreased expression was associated with the susceptible ‘C’ allele of *PSMB8* rs2071464; however, the mechanism is not yet clear. *In silico* prediction tools have predicted that *PSMB8* rs2071464 C>T variation might alter chromatin to enhancer state and result in induced gene expression in peripheral blood cells. Recent studies have explored that several of *cis*-regulatory SNPs could affect histone modifications and change chromatin state transition from repressor to enhancer state [47]. Our results correlate with these findings as higher expression of *PSMB8* was observed in individuals having variant ‘TT’ genotype as compared to ‘CC’ genotype (Fig 1). A significant decrease in transcript as well as protein expression of *PSMB8* in PBMCs of patients with GV and AV is revealed in the present study. Our findings have recently been supported by the blood transcriptomics analysis of vitiligo patients which revealed significant down regulation of *PSMB8* expression in patients [48]. In addition, another recent study has demonstrated the IFN- γ induced lower expression of *PSMB8* in PBMCs of vitiligo patients as compared to controls [34].

Moreover, it has been observed that the down-regulation of *PSMB8* expression leads to suppression of MHC class I molecule surface expression [49]. In addition, the IFN- γ induced immunoproteasomes have been associated with the improved processing of MHC class I antigens [50]. It has been reported that the presentation of a majority of MHC class I epitopes was strikingly reduced in immunoproteasome-deficient mice [51]. Moreover, Xu *et al.*, [52] have also reported a significant decrease of 26S proteasome in lesions of vitiligo patients. Thus, the decreased expression of *PSMB8* in the present study, in conjunction with the above-discussed studies advocates the possibility of reduced MHC class I molecules in the patients and indicates the crucial role of *PSMB8* in vitiligo immunopathogenesis.

Autoimmune diseases are characterized by decreased expression of MHC class I on lymphocytes [53]. The appropriate MHC class I expression is necessary for self-tolerance, and

Table 5. *In silico* prediction results for *PSMB8* rs2071464 polymorphism.

SNP ID	Gene Symbol	SNP Location	Chromosomal Location	Regulome DB Score/ Prediction	HaploRedv4.1 Motifs changed by SNP	Tissue
rs2071464	PSMB8	Intron 6	chr6:32809075	6 / Minimal binding Evidence	7 altered motifs	Peripheral Blood

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Table 6. *In silico* prediction results for *TAP1* rs1135216 polymorphism.

Amino acid change	SIFT	PANTHER	SNPs and GO	POLYPHEN	I-MUTANT	I-MUTANT Score	MUPRO
Asp637Gly	Tolerated	0.34565	Neutral	Benign	Decrease	-1.00	Decrease

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

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abnormalities in such expression may lead to autoimmunity [54]. Zaiss *et al.*, [55] have reported that proteasome immuno-subunits protect against the development of CD8⁺ T-cell mediated autoimmune diseases. They showed that mice deficient for the immune-subunits β5i/LMP7 and β2i/MECL-1 develop early-stage multi-organ autoimmunity following irradiation [55]. Several reports including ours have suggested a decreased CD4⁺/CD8⁺ ratio in vitiligo patients, indicating the prevalence of CD8⁺ cells in patients [56–58]. Thus, a decrease in immunoproteasome levels may lead to a breakdown of self-tolerance, resulting in an increase of CD8⁺ T cells directed towards melanocytes in predisposed individuals which could not be checked upon by the insufficient numbers and functionally deficient regulatory T cells (Tregs) in patients with vitiligo [58,59].

Transport of antigenic peptides across ER membrane is mediated by TAP1 and TAP2 molecules [60]. We did not find a significant association of *TAP1* rs1132516 SNP with vitiligo, as well as there was no difference in *TAP1* transcript levels between cases and controls. The ‘G’ allele occurred predominantly in AV patients compared to SV however, it was considered non-significant due to Bonferroni’s correction. The higher frequency of ‘G’ allele in AV patients indicates its involvement in the autoimmune basis of vitiligo. The bioinformatics analysis revealed that *TAP1* rs1135216 SNP (Asp637Gly) leads to a decrease in the stability of TAP1 protein. Moreover, it has been reported that the polymorphism in *TAP1* gene product did not show any measurable change in protein function but has an influence on peptide selectivity [36]. The binding of antigenic peptides to class I molecules depends on both length (usually 8–10 residues) and sequence [61]. The specificity of these reactions and their biological functions are affected by the 3D conformation of the peptide, HLA complexes, compatibility of the peptide sequence with its HLA class I binding pocket etc [62]. Interestingly, significant differences in the amino-acid signatures of the peptide-binding pockets of MHC class I α chains as well as class II β chains were observed between vitiligo patients and unaffected controls [15]. Though *TAP1* SNP was not associated with vitiligo but the predominant presence of ‘G’ allele in combination with other SNPs in this region might affect the peptide selectivity in patients. *PSMB8* polymorphism in addition to previously reported susceptibility loci such

Table 7. Genetic association studies on *PSMB8* and *TAP1* polymorphisms in Vitiligo.

Sr. No.	Gene	SNP	Population	Association	Reference
1.	<i>PSMB8</i>	rs2071543	Western	No	[14]
		rs2071543	Indian	Yes	[34]
		rs2071627	Western	No	[17]
		rs2071464	Western	Yes	[14]
			Egyptian	No	[44]
			Saudi	No	[22]
			Western	No	[14]
2.	<i>TAP1</i>	Intron7 C/T	Western	Yes	[14]
			Saudi	Yes	[22]
		rs1135216	Saudi	Yes	[45]

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TNFA, *TNFB*, *IL1B*, *IFNG*, *NALP1*, *IL4* etc. demonstrate immunogenetic predisposition in vitiligo patients from Gujarat [7, 63–67]. Overall, studies implicate a break in immunological tolerance in vitiligo. A similar type of etiopathology has been observed in alopecia areata (a common autoimmune disorder that often results in unpredictable hair loss). The melanocyte is the main autoimmune target in both the disorders. Both are IFN- γ dependent and shares common immunogenetic loci such as *AIRE*, *CTLA4*, *NALP1*, and MHC region [68–72]. Surprisingly, the co-occurrence of vitiligo and alopecia areata is rare [73]. Unequal expression of MHC class I and II might be a base for the reverse correlation between the incidents of vitiligo and alopecia areata [73]. Hence, the genes involved in antigen processing might have a role in the breakdown of immune tolerance and precipitation of vitiligo.

Conclusion

In conclusion, the association of *PSMB8* rs2071464 polymorphism with generalized and active vitiligo suggests defective antigen processing which might influence the peptide repertoire presented to the immune cells targeting melanocytes. However, further replicative studies and *in vitro* functional studies for *PSMB8* and *TAP1* are needed to delineate the role of defective antigen processing and presentation pathways in vitiligo pathogenesis.

Supporting information

S1 Text. Bioinformatics analysis.

(DOC)

S1 Table. Demographic characteristics of patients with vitiligo and controls.

(DOCX)

S2 Table. Primers used for genotyping of *PSMB8* rs2071464 and *TAP1* rs1135216 SNPs.

(DOCX)

S3 Table. Primers used for Sequencing of *PSMB8* and *TAP1* SNPs.

(DOCX)

S4 Table. Primers used for gene expression of *PSMB8* and *TAP1*.

(DOCX)

S1 Fig. (A) PCR-RFLP analysis of *PSMB8* rs2071464 SNP on 3.5% agarose gel: Lane M shows 50bp DNA ladder, lanes: 1 & 2 show homozygous (CC) genotypes; lanes: 3 & 6 show homozygous (TT) genotypes and lanes: 3 & 6 show heterozygous (CT) genotypes. (B) ARMS-PCR analysis of *TAP1* rs1135216 SNP on 3.5% agarose gel: Lane M shows 50bp DNA ladder, lanes: 1, 2 & 3, 4 show homozygous (AA) genotypes; lanes: 5, 6 shows heterozygous (AG) genotype and lanes: 7, 8 shows homozygous (GG) genotype.

(TIF)

S2 Fig. Confirmation of genotyping results of *PSMB8* rs2071464 SNP by sequencing of PCR products. A) *PSMB8* rs2071464 CC genotype, B) *PSMB8* rs2071464 CT genotype, C) *PSMB8* rs2071464 TT genotype.

(TIF)

S3 Fig. Confirmation of genotyping results of *TAP1* rs1135216 SNP by sequencing of PCR products. A) *TAP1* rs1135216 AA genotype, B) *TAP1* rs1135216 AG genotype, C) *TAP1* rs1135216 GG genotype.

(TIF)

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