Proteasome subunit beta type-8 (PSMB8) gene Polymorphisms in Vitiligo: A possible predictor of auditory involvement

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Abstract

Introduction: Proteasome subunit beta type-8 (PSMB8) is a protein that contributes to

the complete assembly of 20 S proteasome complexes which play a role in the pathogenesis of vitiligo.

Aim of the work: The study aimed to evaluate the association between PSMB8 gene polymorphisms with vitiligo to assess its clinical significance among an Egyptian sample of vitiligo patients.

Subjects and methods: Genomic DNA was isolated from blood samples of 100 vitiligo patients and 100 control subjects and detection of PSMB8 polymorphisms was done by Real Time PCR. Data analysis was carried out for the entire cohort. Statistics were performed using software. Audiological evaluation including pure-tone audiometry, extended high-frequency audiometry, transient evoked otoacoustic emissions, and auditory brainstem response was carried out.

Results: There was a significant difference between PSMB8 genotypes and alleles distribution in patients and control groups. Ten percent of the study sample had sensorineural hearing loss. The patients with hearing loss were significantly older (p=0.0002), had significantly later age of onset (p=0.0007), longer duration (p=0.0021), higher BMI (p=0.045) and higher VASI scores (p=0.0015). All of them suffered from extensive forms of vitiligo (generalized and universal). Regarding the VIT rs2071543 polymorphism, all of vitiligo patients with hearing loss were carrying the CA and AA genotypes. None of them carried the reference genotype; CC. The A allele of VIT rs2071543 was significantly associated with hearing affection (p=0.024)

Conclusion: PSMB8 polymorphism was associated with the susceptibility to develop vitiligo and has clinical significance among the studied group of Egyptian patients. Factors predicting auditory abnormalities should be further studied for early detection

and management.

Introduction

Vitiligo is a disfiguring depigmenting disease that results from the absence of melanocytes in the affected areas **(1).** Many theories have been proposed to explain this melanocytes absence. These theories included immunological, genetic, neural, cytotoxic, melanocytorrhagy and oxidative stress ones **(2).**

Proteasome subunit beta type-8 (also known as 20S proteasome subunit iβ5) is the constitutive proteasome which represents the proteolytic portion of the 26S proteasome (immunoproteasome). Antigen presenting cells show abundant 26S proteasomes expression **(3),** where it performs an essential role in antigen processing and presentation to cytotoxic T lymphocytes via major histocompatibility complex (MHC) class I molecules **(4)**. Moreover, it has a regulatory effect on the T cell polarization and macrophage activation with subsequent involvement in the etiopathogenesis of many autoimmune inflammatory disorders **(5)**. Serum proteasomes may be considered as inflammatory markers in certain autoimmune diseases e.g. systemic lupus erythematosus, Sjogren's syndrome and rheumatoid arthritis **(6).**

Polymorphisms of PSMB8 gene are involved in the development of some auto-inflammatory disorders such as Nakajo-Nishimura syndrome **(7)** and chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome **(8)**. These gene polymorphisms have been also studied in vitiligo in an Indian sample of patients **(9, 10)**, however, it has not been investigated enough in different ethnic populations.

The current study aimed to investigate the association between PSMB8 (rs2071543 and the 6 intron at 37360) gene polymorphisms in a sample of Egyptian vitiligo patients and to assess the association between different genotypes and the clinical aspects of the disease.

Subjects and Methods:

The study included 100 patients suffering from different clinical presentations of vitiligo, in addition to 100 apparently healthy vitiligo free individuals of matched age, sex and body mass index (BMI) as a control group. All participants were recruited from the Outpatient Clinic of the Dermatology Department of Benha University after approval of the study by the Ethics Committee on research involving human subjects. Informed consents were obtained from all participants prior to the study beginning.

All patients were subjected to full history taking and dermatological examination to assess the distribution and the extent of the vitiliginous patches. Vitiligo severity was evaluated using Vitiligo Area Severity Index (VASI) **(11)**.

Audiological evaluation including pure-tone audiometry, extended high-frequency audiometry, transient evoked otoacoustic emissions, and auditory brainstem response was carried out. Eye examination including visual acuity, intraocular tension, and fundus examination was carried out.

DNA extraction and Genotyping

Peripheral blood samples from all participants were extracted in a tube with EDTA, and genomic DNA was isolated with GeneJET Genomic DNA Purification Kit cat. No K0721 (Qiagen- Germany). After extraction, the concentration

and purity of genomic DNA were quantified spectrophotometrically by UV absorbance using Nanodrop (Thermo Scientific, Wilmington). The quality of DNA was assessed with the A260/280 ratio; a value of 1.8 - 2 was considered of good quality. Allelic discrimination was performed using the Taqman SNP ready-made assay (Qiagen-Germany) which includes TAQMAN UNIVERSAL MMIX II (Cat. NO 4440043) and TAQMAN SNP ASSAYS MTO HUMAN SM (Cat. NO 4351379). The TaqMan genotyping assay (40X) consisted of forward primer, reverse primer and two probes. The forward primers sequences were5'TCCCTAGGGGCTTCCCTACTGC-3 and 5'TTGATTGGCTTCCCGGTACTG-3; and the reverse primers sequences were 5'-TCGATCTGTGGCTTTCGCTTTC-3 and 5'TCTACTACGTGGATGAACATGG-3 for PSMB8 rs2071543and the six intron at 37360 site respectively. The sequences of the two probes were VIC-ACTCCTTTCACCTATTCCCAAGGCCT-MGB-NFQ (normal probe 5′-3′) and FAM-CAGCTACACCTGTATGTAGGCTAGA MGB-NFQ (mutant probe 5′-3′).

The probes were designed with minor groove binder (MGB) and non-fluorescent quencher (NFQ) at the 3′ end, whereas the 5′ end contained the fluorescence reporter dyes 2′-chloro 7′-phenyl-1,4-dichloro-6-carboxyfluorescein(VIC) or 6 carboxyfluorescein (FAM). The wild type probe labeled with VIC dye while the variant probe labeled with FAM dye. Real-time PCR was performed in a volume of 20$_u$l using Rotorgene real time PCR system *(Qiagen- S.Korea)*. The reaction was set with 1X TaqMan Universal Master Mix, 1X Taqman assay and the reaction volume was completed by nuclease free water. Thermal cycling conditions were as following: 60C for 30 sec, 95C for 10 min, 40 cycles of denaturation 95°C for 15 sec and annealing/extension 60°C for 1 min.

Statistical Analysis

Data were tabulated and analyzed using the statistical package SPSS (Statistical Package for the Social Sciences) version 25. Data were summarized using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests. For comparing categorical data, Chi square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is less than 5. Genotype and allele frequencies were compared between the disease and the control groups. Odds ratio (OR) with 95% confidence intervals was calculated using binary logistic regression. P-values less than 0.05 were considered as statistically significant.

Results

The mean age of the studied groups was 23.79 ± 15.87 years for the patients group and 24.46 ±9.12 years for the control group. Sixty eight percent of the patients and 60% of the control subjects were males. There was insignificant differences between cases and control subjects regarding age and sex (p=0.147 and 0.332 respectively). Both groups were matching regarding body mass index, 24.41 ± 5.57 kg/m^2 in patients and 25.49 ± 4.40 kg/m^2 in the control group (p= 0.094).

The mean disease duration was 6.68 ± 6.39 years. Fifty one percent of patients reported stress as a provocative factor for the disease and 36% only gave a positive history of Koebnerization. Hearing was impaired in 10% of patients, while vision was impaired in only 8%. Segmental vitiligo represented 13% only of the patients. Leukotrichia was observed in 37 (37%) cases only. The mean VASI score was 13.16 ± 24.16.

Applying Hardy Weinberg equation, revealed that PSMB8 (rs2071543) and (intron6snp) genotypes and alleles frequency in patients and control groups were in Hardy Weinberg equilibrium **(Table 1)**.

Table (1): Hardy Weinberg equilibruim of the studied SNPs.

		Cases(N=100)	**Control (N=100)**
		Count (%)	**Count (%)**
rs2071543	**AA**	52(52.0)	12(12.0)
	CA	35(35.0)	28(28.0)
	CC	13(13.0)	60(60.0)
	allele A	139(69.5)	52(26.0)
	allele C	61(30.5)	148(74.0)
	HW *p*	0.081	0.0541
intron6snp	**TT**	48(48.0)	10(10.0)
	GT	37(37.0)	28(28.0)
	GG	15(15.0)	62(62.0)
	allele T	133(66.5)	48(24.0)
	allele G	67(33.5)	152(76.0)
	HW *p*	0.090	0.100

HW***p***:Hardy Weinberg equilibrium**;** A:adenine; C:cytosine; G: Guanine; T: Thymine.

AA, CA genotypes and A allele of PSMB8 (rs2071543) SNP and TT, GT genotypes and T allele of PSMB8 (intron6snp) SNP increase the risk of vitiligo development. The combined polymorphisms at both studied SNPs increases the risk of vitiligo development about 20 folds **(Table 2).**

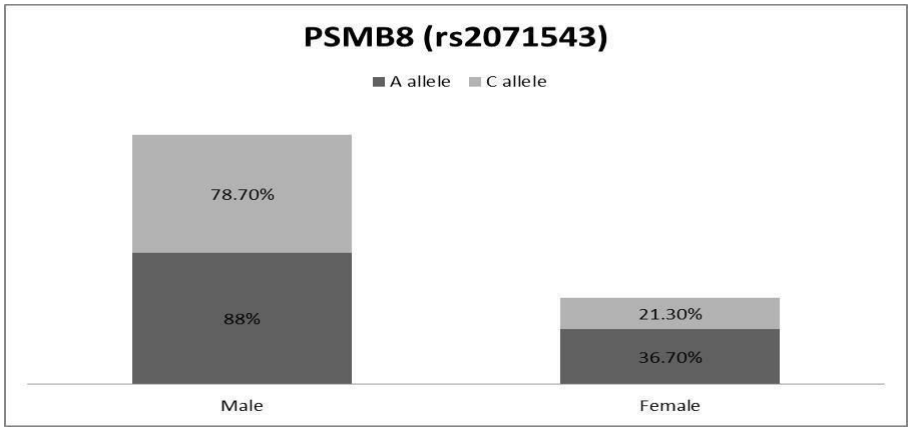
Table (2): Genotypes and alleles of the studied SNPs**.**

	Cases (N=100)	Control (N=100)	P value	OR	95% CI	
	Count (%)	Count (%)			lower	Upper
PSMB8(rs2071543) genotypes and alleles						
AA	52(52.0)	12(12.0)	<0.001	20.000	6.883	58.110
CA	35(35.0)	28(28.0)	<0.001	5.769	2.349	14.172
CC	13(13.0)	60(60.0)	Reference			
AA+CA	87(87.0)	40(40.0)	<0.001	10.038	4.456	22.617
allele A	139(69.5)	52(26.0)	<0.001	6.485	3.784	11.115
allele C	61(30.5)	148(74.0)	Reference			
PSMB8(intron6snp) genotypes and alleles						
TT	48(48.0)	10(10.0)	<0.001	19.840	6.550	60.100
GT	37(37.0)	28(28.0)	<0.001	5.462	2.287	13.047
GG	15(15.0)	62(62.0)	Reference			
TT+GT	85(85.0)	38(38.0)	<0.001	9.246	4.187	20.414
allele T	133(66.5)	48(24.0)	<0.001	6.286	3.646	10.839
allele G	67(33.5)	152(76.0)	Reference			
Haplotype						
rs2071543 allele A+ intron6snp allele G	35(17.5)	32(16.0)	<0.001	4.102	1.975	8.517
rs2071543 allele A+ intron6snp allele T	104(52.0)	20(10.0)	<0.001	19.500	8.958	42.450
rs2071543 allele C+ intron6snp allele T	29(14.5)	28(14.0)	0.001	3.884	1.801	8.378
rs2071543 allele C+ intron6snp allele G	32(16.0)	120(60.0)	Reference			

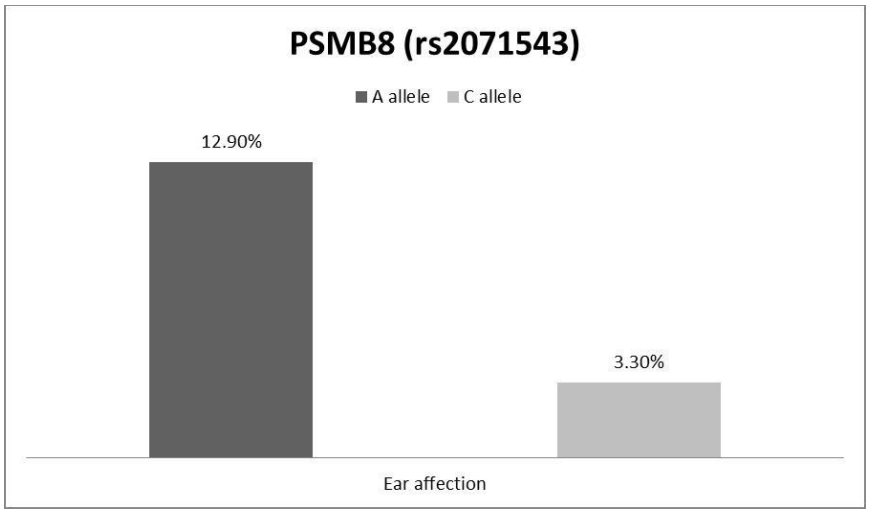
OR:Odds ratio; A:adenine; C:cytosine; G: Guanine; T: Thymine.

Among the studied criteria, the studied SNPs genotypes and alleles distribution didn't show any significant difference except for the predominance of A allele of rs2071543 in

female patients and in patients with ear affection **(Graphs 1 and 2)**



Graph 1: PSMB8 (rs2071543) alleles gender difference



Graph 2: PSMB8 (rs2071543) alleles in patients with ear affection

Features of patients with auditory involvement

Audiological evaluation of the studied patients revealed sensorineural hearing loss in 10% of the study sample (10 patients). The patients with hearing loss were significantly older when compared to those without hearing loss (p=0.0002). They also had significantly later age of onset (p=0.0007), longer disease duration (p=0.0021) and higher BMI (p=0.045). Auditory dysfunction was associated with significantly higher VASI

scores (p=0.0015). All of the patients who had hearing loss suffered from extensive forms of vitiligo (generalized and universal). Regarding the PSMB8 rs2071543 polymorphism, all of vitiligo patients with hearing loss were carrying the CA and AA genotypes. None of them carried the reference genotype; CC. The A allele of PSMB8 rs2071543 was significantly associated with hearing affection (p=0.024) **(Table 3)**,

Table 3: Comparison between patients with and without hearing affection.

The variables		Patients with hearing affection n=10	Patients without hearing affection n=90	p
		mean±SD	mean±SD	
Age		40.2±18.9	21.3±14.21	0.0002
Age of onset		28.2±18	13.29±12.12	0.0007
Disease duration		12.4±5.1	5.96±6.1	0.0021
BMI		27.69±2.8	24.04±5.6	0.045
VASI		35.43±39.1	10.68±20.27	0.0015
The variables		**N (%)**	**N (%)**	**p**
Gender	Male	9 (90)	59 (65.6)	0.12
	Female	1 (10)	31 (34.4)	
Family history	Yes	3 (30)	31 (34.4)	0.78
	No	7 (70)	59 (65.6)	
Type	SV	0 (0)	13 (14.4)	0.35
	NSV	10 (100)	77 (85.6)	
Types	Acrofacial	0 (0)	52 (57.8)	0.0004
	Generalized and Universal	10(100)	25(27.8)	
Leukotrichia	Yes	4 (40)	33 (36.7)	0.84
	No	6 (60)	57 (63.3)	
Response to treatment	Responsive	4 (40)	48 (53.3)	0.42
	Resistant	6 (60)	42 (46.7)	
PSMB8 gene (rs2071543)	CC	0 (0)	13 (14.5)	0.3
	CA+AA	10 (100)	77 (85.5)	
	C allele	2	59	0.035

	A allele	18	121	
PSMB8 gene (intron6snp)	GG	3 (30)	12 (13.3)	0.16
	GT+TT	7 (70)	78 (86.7)	
	G allele	9	58	0.25
	T allele	11	122	

Discussion

Two SNPs may occur at PSMB8 gene; the rs2071543 and intron6snp SNPs. The amino acid changes resulting from gene polymorphisms reduce immunoproteasome activity, with subsequent induction of inflammatory innate immune responses and production of reactive oxygen species (ROS) **(12).**

In the present study, the association of these SNPs with vitiligo in an Egyptian sample of vitiligo patients was studied. Our findings revealed that AA and CA genotypes and A allele of PSMB8 gene (rs2071543) as well as TT and GT genotypes and T allele of PSMB8 gene (intron6snp) increase the risk of vitiligo significantly. The same result was found by **Dani et al. (10)** in an Indian sample of vitiligo patients**.** However, they didn't report significant difference between patients and controls regarding GT genotype.

The association between PSMB8 2 gene polymorphisms and vitiligo development was further strengthened by the haplotype analysis which revealed that the presence of A allele in (rs2071543) increases the risk of vitiligo by 4 folds, the presence of T allele in (intron 6snp) increases the risk of vitiligo by 4 folds, while the combined A allele at (rs2071543) and T allele at (intron 6snp) increase the risk of vitiligo by 20 folds.

Casp et al. (13) found that the T allele of PSMB8 (intron 6snp) had a protective role against vitiligo development. While the present study found that T allele of the same SNP was a risk factor for vitiligo development. This difference is not completely understood

as the effect of T allele in this position on the PSMB8 activity or serum levels is not clear yet. However, this difference could be attributed to the different ethnicity of the studied patients in both studies.

A allele of rs2071543 was predominant in female patients in this study, while there was insignificant difference between males and females regarding genotypes distribution. However, **Dani et al. (10)** found that A allele and genotype CA were significantly increased in male patients. In fact, proteasome activity may be affected by gender difference **(14)**, and many genes polymorphisms and genetic susceptibility may differ from men to women **(15)**, so further studies would be of importance regarding the gender differences in the studied gene polymorphisms.

There is a convincing evidence that vitiligo is a systemic disorder influencing the whole pigmentary system, including melanocytes in the inner ear. Cochlear melanocytes and also melanin-containing cellular elements of the auditory system may be affected in vitiligo with subsequent interference with the conduction of action potentials **(16)**. There are discrepancies in the literature about the specific influence of vitiligo on auditory threshold. Some authors **(17, 18)** reported vitiligo associated hearing affection, while others **(19)** reported no hearing affection in vitiligo patients.

Many theories were advocated to explain the role of cochlear melanocytes in normal hearing. None of them is well established, but they all share the final step; the disappearance of cochlear functioning melanocytes leads to the loss of the supporting and protective effects of melanin. This may contribute to the impaired hearing acuity **(20).**

In the present study, 10% of our vitiligo patients had impaired hearing acuity. In those

patients, A allele of (rs2071543) was significantly more predominant. In spite of this observation, the current workers propose that vitiligo patients carrying A allele at (rs2071543) require routine monitoring for auditory and vestibular functions for early identification and management of auditory changes as the disease progresses.

In the current study, vitiligo patients with hearing abnormality were significantly older and reported longer disease duration when compared to those without hearing deficits. This comes in agreement with **Ardie et al. (21)** and **Aslan et al. (22)** who reported a significant relation between the duration of vitiligo and hearing loss. In fact, the progressive loss of cutaneous melanocytes may be associated with a progressive loss of cochlear melanocytes leading to a continuous deterioration of hearing acuity over time. However, the possibility of deterioration of hearing acuity as a manifestation of normal ageing cannot be excluded. So more studies may be helpful to clarify this association.

On the other hand, **Sharma et al. (23), Elsaied et al. (19)** and **Mahdi et al. (24)** didn't report a relation between the hearing abnormalities in vitiligo patients and their age or their disease duration. They all proposed a theory that the pigment cells in the ear are affected at the disease onset then they stabilize. This discrepancy in the results may be explained by the significantly older age and longer disease duration in our patients when compared to the aforementioned studies.

The mean age of onset in our patients with auditory problems was significantly higher when compared to those who didn't show auditory defects (28.2±18 vs 13.29±12.12 years old). **Al-Mutairi and Al-Sebeih (25)** concluded that no significant relation was detected between late onset vitiligo and auditory disorders; however they didn't compare the incidence of hearing impairment between early and late onset vitiligo cases.

The mean BMI of our hearing affected patients was significantly higher than that in the other group. To the best of our knowledge, this relation was not highlighted before, however, **Hu et al. (26)** concluded that increased BMI may carry an increased risk of hearing impairment especially in cases of morbid obesity.

It was interesting to find out that all our patients with hearing loss suffered from extensive forms of vitiligo (generalized or universal vitiligo). We think that extensive cutaneous involvement suggests an aggressive pathogenic process which may destroy ear pigment cells. However, **Moghaddam et al. (27)** concluded that there was no significant association between vitiligo severity and the auditory defects. Moreover, hypoacusis patients in **Mutairi and Al-Sebeih (25)** study were distributed on different vitiligo forms. This discrepancy of findings may be a nucleus for more research.

As not all vitiligo patients will experience auditory problems, and most of the hearing affected patients may be clinically asymptomatic at the beginning, it would be beneficial to study the factors which may be associated with auditory involvement in vitiligo patients to determine the patients at higher risk in order to start auditory monitoring and intervention as early as possible **(20)**.

Conclusion

PSMB8 (rs2071543) and (intron6snp) gene polymorphisms are associated with vitiligo susceptibility and they may alter the clinical aspects of the disease. Older vitiligo patients, and those with later age of disease onset, longer disease duration, higher BMI and higher VASI scores are at higher risk to develop auditory impairment. Factors predicting auditory abnormalities should be studied carefully to allow early detection and

management of this problem.

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