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Report

Analysis of the heat shock protein 70 (HSP70) genetic variants in nonsegmental vitiligo patients

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Abstract

Background Vitiligo is an autoimmune disease that courses with skin depigmentation because of the destruction of melanocytes. Vitiliginous melanocyte is prone to damage because of oxidative stress which activates cellular stress response and the release of heat shock proteins such as HSP70 promoting immune activation against the melanocyte. Variants in HSP70 genes (HSPA) might alter their expression and thus modulate vitiligo susceptibility. Therefore, we sought to evaluate the role of the 5′ untranslated region HSPA1A G/C (rs1043618) and the exonic HSPA1B A/G (rs1061581) and HSPA1L T/C (rs2227956) gene variants in nonsegmental vitiligo.

Methods A total of 200 nonsegmental vitiligo patients and 208 age/gender-matched healthy subjects were genotyped for rs1043618, rs1061581, and rs2227956 variants by PCR-RFLP.

Results Variants rs1043618 and rs1061581 were not associated with vitiligo susceptibility. On the other hand, the rs2227956 C allele and TC genotype were associated with protection against vitiligo. A similar effect was observed for the GAC haplotype. Any of the aforementioned HSP70 gene variants were associated with the clinical characteristics of vitiligo.

Conclusion Our findings suggest that the HSPA1L rs2227956 gene variant might influence the susceptibility to vitiligo. Being the first study of HSP70 gene variants in vitiligo, further research is encouraged to corroborate these results.

Introduction

Vitiligo is the most common acquired pigmentation disorder which is characterized by the appearance of white macules on the skin caused by the loss of epidermal melanocytes or their function.[1,2] Plenty of evidence indicates that melanocyte destruction is immune mediated, thus vitiligo is widely accepted as an autoimmune disease with several immune components already identified as majorly implicated in its pathogenesis.[1,3]

The initial trigger of vitiligo appears to be melanocyte stress which contributes to the initiation of autoimmunity through neoantigen formation and activation of innate immunity mediated by the action of stress-induced molecules such as heat shock proteins (HSPs).[3]

In this respect, it has been documented that vitiliginous melanocytes preferentially secrete the inducible form of heat shock protein 70 (HSP70) in response to stress.[4] This secretory HSP70 form is bound to melanocyte antigens and released to the intercellular milieu as a damage-associated molecular pattern (DAMP) capable of inducing activation of dendritic cells initiating and/or precipitating the autoimmune response against the melanocyte.[5,6] Because of these characteristics, HSP70 is

now considered a central actor in vitiligo pathogenesis, and its blocking has already proven a promising approach for preventing and treating vitiligo.[5,7] However, the implication of HSP70 encoding genes has not yet been explored.

The three main HSP70 genes, HSPA1A, HSPA1B, and HSPA1L are located in the HLA class III region in chromosome 6p21.3.[8] Both HSPA1A and HSPA1B encode a similar inducible HSP70, whereas HSPA1L encodes a not inducible, constitutive HSP70 which shares high homology with the inducible form.[9,10] Three single nucleotide variants (SNVs) have been extensively studied for these genes: HSPA1A rs1043618, HSPA1B rs1061581, and HSPA1L rs2227956. The first is a G/C transversion in the 5′ untranslated region (UTR); the second, a silent exonic A/G transition; and the third, an exonic T/C transition which changes the methionine at position 493 of the peptide for a threonine.[8,11,12]

Although these SNVs have not been explored in vitiligo, they have been reported associated with autoimmune disorders such as pemphigus foliaceus, multiple sclerosis, Graves' disease, and spondyloarthropathy.[8,9,13,14]

Considering the aforementioned role of HSP70 SNVs in the gene/protein expression and/or its functionality along with their reported association with other autoimmune diseases, here, we aimed to assess the association of HSPA1A G/C (rs1043618), HSPA1B A/G (rs1061581), and HSPA1L T/C (rs2227956) SNVs in vitiligo and its clinical characteristics.

Material and methods

Subjects

The present case–control study was performed in Culiacan General Hospital, Sinaloa, Mexico, under the approval of the local Ethics Committee. Procedures were in agreement with the Declaration of Helsinki of 1975, as revised in 1983.

Study participants were recruited according to the following criteria. The patient group included 200 unrelated individuals presenting a definitive clinical diagnosis of nonsegmental vitiligo made by dermatologists based on the classification of the Vitiligo Global Issues Consensus Conference, Bordeaux, France, 2011.[2] Clinical variables of relevance (clinical type, age of onset, disease activity, and family history of vitiligo) were collected via questionnaire. Individuals with other autoimmune diseases (Hashimoto's thyroiditis, Graves' disease, psoriasis, rheumatoid arthritis, ankylosing spondylitis, lichen planus, and thrombocytopenic purpura) were also considered in the patient group. The control group was composed of 208 age–gender-matched healthy individuals without a family history of vitiligo or other autoimmune diseases.

Sociodemographic variables and written informed consent were obtained from all the participants.

Genotyping

Peripheral blood was collected in EDTA-K_3 anticoagulant-containing tubes to later isolate DNA through the DTAB-CTAB protocol.[15] Genotyping was performed using the conditions previously described by Toumi et al.[9] Briefly, polymerase chain reaction (PCR) was done using the forward 5′-GAGAGTGACTCCCGTTGTCC-3′ and reverse 5′-GAGTAGGTGGTGCCCAGGT-3′ primers for HSPA1A G/C (rs1043618); 5′-CATCGACTTCTACACGTCCA-3′ and 5′-CAAAGTCCTTGAGTCCCAAC-3′ for HSPA1B A/G (rs1061581); and 5′-GGACAAGTCTGAGAAGGTACAG-3′ and 5′-GTAACTTAGATTCAGGTCTGG-3′ for HSPA1L T/C (rs2227956) variants, with annealing temperatures of 65, 56, and 55°C, respectively. The variants were detected by restriction fragment length polymorphism (RFLP) using the restriction enzymes *Bsr*BI for rs1043618, *Pst*I for rs1061581, and *Nco*I for rs2227956, and visualized in agarose gels (Fig. 1). Additionally, 5% of the genotyped samples were confirmed by Sanger DNA sequencing (Macrogen, Inc., Seoul, Korea).

Statistical analysis

Case–control genetic association analysis was performed using conditional logistic regression to obtain age–gender adjusted odds ratios (ORs) and 95% confidence intervals (CIs). Association between the polymorphisms and clinical variables was done with binary logistic regression. The aforementioned analyses were performed in Statistic Package of Social Sciences (SPSS) v.20, taking p values <0.05 as statistically significant. Additionally, Hardy–Weinberg equilibrium was evaluated using DeFinetti software (https://ihg.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl) and haplotype and linkage disequilibrium analysis with SHEsisPlus online platform (http://shesisplus.bio-x.cn/SHEsis.html).[16] Finally, statistical power was 90% according to G*Power v.3.1.9.7 software calculations given the study sample size ($n = 408$) considering a two-tailed hypothesis and OR = 1.5.

Results

The clinical and sociodemographic features of the study participants are shown in Table 1. Genotype frequencies of HSPA1A rs1043618, HSPA1B rs1061581, and HSPA1L rs2227956

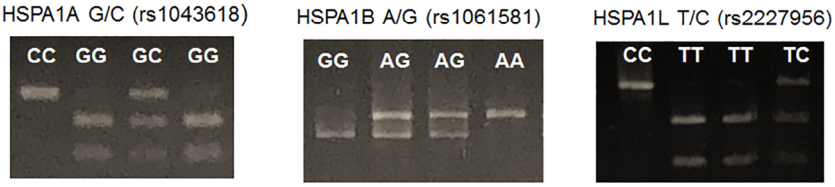

Figure 1 Agarose gel visualization of the HSP70 genetic variant genotypes

Table 1 Clinical and sociodemographic characteristics of the study participants

Characteristic		Vitiligo *n* = 200 (%)	HCs *n* = 208 (%)
Age[a]		44 (27–54)	40.5 (26–51.75)
Gender	Male	96 (48)	104 (50)
	Female	104 (52)	104 (50)
Family history of vitiligo		114 (57)	
Age of onset[a]		24 (12–38)	
Clinical type	Focal[b]	12 (6)	
	Acrofacial	21 (10.5)	
	Generalized	153 (76.5)	
	Universal	14 (7)	
VIDA score	−1	11 (5.5)	
	0	68 (34)	
	1	59 (29.5)	
	2	23 (11.5)	
	3	31 (15.5)	
	4	8 (4)	
Koebner phenomenon		48 (24)	
Other autoimmune disease[c]		35 (17.5)	

HCs, healthy controls; VIDA, vitiligo disease activity.
[a]Values expressed as median (25th-75th percentile).
[b]Bilateral, symmetrical lesions (i.e., both eyelids or belt line).
[c]Included: Hashimoto thyroiditis (*n* = 18), Graves' disease (*n* = 6), psoriasis (*n* = 4), rheumatoid arthritis (*n* = 4), ankylosing spondylitis (*n* = 1), lichen planus (*n* = 1), and thrombocytopenic purpura (*n* = 1).

variants were in agreement with Hardy Weinberg equilibrium in both vitiligo and healthy control (HC) groups. There were no differences in the frequencies of rs1043618 and rs1061581 between vitiligo patients and HCs (Table 2). On the other hand, because of the low frequency of rs2227956 CC genotype (minor allele homozygous), the analysis was performed following a dominant genetic model in which the TC + CC category and the C allele were associated with protection against vitiligo (OR = 0.512 and 0.596, respectively, Table 2).

Further analysis of the HSP70 genetic variants indicated linkage disequilibrium between rs1043618, rs1061581, and rs2227956 according to D′ values (>0.8), although R^2 values only supported it between the first two (Fig. 2).

In accordance with the genotype association tests, haplotype analysis showed that individuals bear five major haplotypes of which GAC, the only having the rs2227956 C allele, was associated with a decreased risk of developing vitiligo (OR = 0.497, Table 3).

Finally, we did not find differences in the allele, genotype, or haplotype frequencies of the studied HSP70 variants between the categories of the clinical characteristics of vitiligo listed in Table 1 (data not shown).

Discussion

The role of HSP70 has been previously studied in vitiligo by different approaches, suggesting its participation in disease development through induction of the inflammatory response and melanocyte-reactive T lymphocytes.[1,3,4,7,17] Despite the latter, HSP70 gene variants have not been yet studied in vitiligo but have been associated with other autoimmune diseases.[8,9,13,14] Therefore, the results of the present study will be discussed in light of those associations.

The HSPA1A rs1043618 and HSPA1B rs1061581 SNVs have been previously associated with a reduction of gene/protein expression of the inducible HSPA1A and HSPA1B, respectively.[8,11] Using an age–gender adjusted approach, we did not find any association of these variants with vitiligo (Table 1), suggesting that the participation of inducible HSP70 in disease pathogenesis might not be influenced by SNVs in its coding

Table 2 Allele and genotype frequencies of the studied HSP70 gene variants in vitiligo patients and healthy controls

Variant		Vitiligo *n* = 200 (%)	HCs *n* = 208 (%)	*P*	OR (95% CI)[a]
HSPA1A G/C	GG	95 (47.5)	105 (50.5)	-	1.0 (reference)
rs1043618	GC	87 (43.5)	80 (38.5)	0.400	1.203 (0.782–1.849)
	CC	18 (9)	23 (11)	0.606	0.837 (0.426–1.645)
	C	0.307	0.303	0.983	1.003 (0.783–1.283)
HSPA1B A/G	AA	46 (23)	49 (23.6)	-	1.0 (reference)
rs1061581	AG	108 (54)	115 (55.3)	0.989	0.997 (0.634–1.567)
	GG	46 (23)	44 (21.1)	0.749	1.094 (0.631–1.896)
	G	0.500	0.488	0.232	0.766 (0.495–1.186)
HSPA1L T/C	TT	180 (90.5)	171 (82.6)	-	1.0 (reference)
rs2227956[b]	TC + CC[c]	19 (9.5)	36 (17.4)	0.024	0.512 (0.286–0.917)
	C	0.047	0.092	0.043	0.596 (0.361–0.985)

HCs, healthy controls; OR (95% CI), odds ratio (95% confidence interval).
[a]Adjusted for age and gender.
[b]Vitiligo *n* = 199, HCs *n* = 207.
[c]HSPA1L CC genotype *n*: vitiligo = 0, HCs = 2.

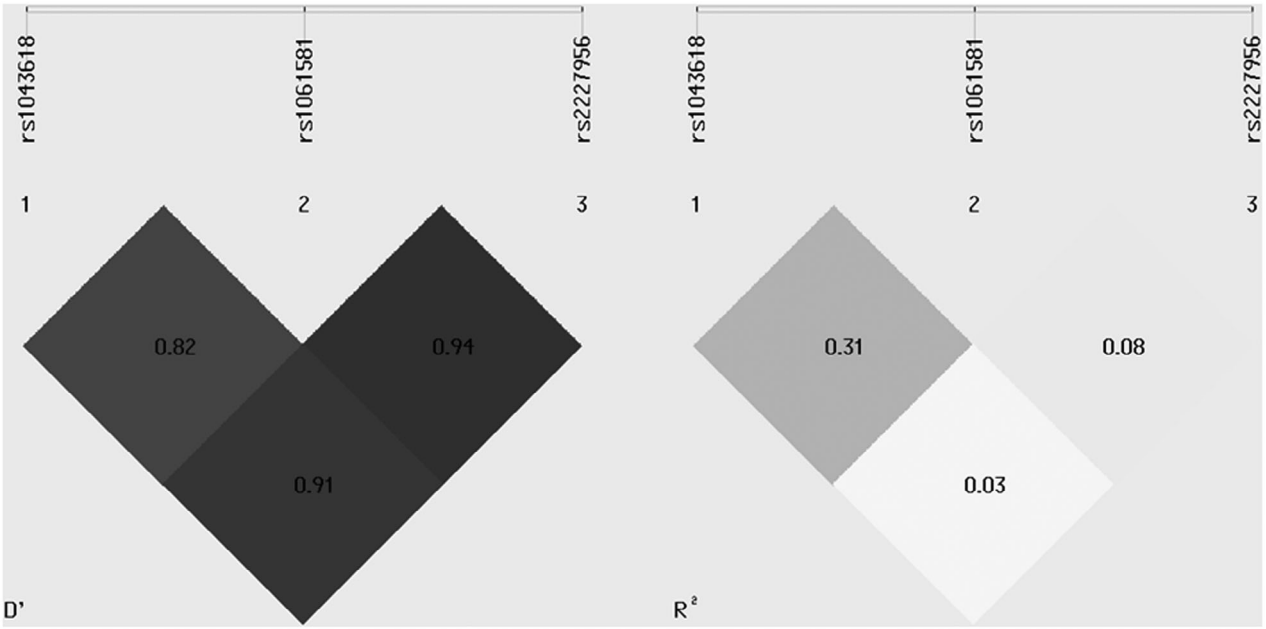

Figure 2 Linkage disequilibrium test of HSP70 gene variants

Table 3 Haplotype frequencies of the studied HSP70 gene variants in vitiligo patients and healthy controls

Haplotype HSPA1A,B,L	Vitiligo	HCs	*P*	OR (95% CI)
G A T	0.445	0.398	0.161	1.219 (0.923–1.611)
C G T	0.297	0.277	0.506	1.108 (0.818–1.501)
G G T	0.200	0.207	0.811	0.959 (0.682–1.349)
G A C	0.045	0.086	0.017	0.497 (0.277–0.891)
C A T	0.010	0.024	0.176	0.410 (0.127–1.318)

HCs, healthy controls; OR (95% CI), odds ratio (95% confidence interval).

genes. It is important to mention that our results contrast with previous reports of an increased risk of disease associated with HSPA1A rs1043618 C allele in pemphigus foliaceus and with HSPA1B rs1061581 GG genotype and G allele in multiple sclerosis, Graves' disease, spondyloarthropathy, and also pemphigus foliaceus,[8,9,13,14] differences that could be attributable to particularities of the studied diseases and/or population genetics, thus encouraging further investigations to better address these HSP70 SNV effects in autoimmunity, but particularly in vitiligo.

On the other hand, we found a link of HSPA1L rs2227956 rare C allele with protection against vitiligo (see Table 2). A similar relationship was reported in pemphigus foliaceus and spondyloarthropathy, implying a role of the rs2227956 C allele in the protection to develop autoimmune pathologies.[9,14] This finding could be attributable to the hypothesized decrease in HSP70-protein interaction caused by the change of the nonpolar amino acid methionine (T allele) for the polar threonine (C allele) at position 493, located in the peptide-binding domain of the protein,[12,18] which would render HSP70 less avid to chaperone self-antigens to be presented by the major histocompatibility complex (MHC) molecules.[19] However, additional bioinformatic analysis performed in SIFT (https://sift.bii.a-star.edu.sg) and ISLAND (https://island.pythonanywhere.com/welcome/default/index) suggests that the amino acid substitution in HSPA1L protein has no impact in neither its functionality nor its binding affinity to melanocyte antigens (TRP-1, TRP-2, tyrosinase, MART-1, and PMEL), respectively (data not shown). Therefore, the protective effect of rs2227956 C allele against vitiligo is more likely related to its association with a decreased production of HSP70 protein in monocytes and lymphocytes,[12] and with lower HSPA1L gene expression in skin,[20] events that could attenuate its DAMP function reducing the risk of mounting a pathogenic autoreactive immune response.[1,3,6]

It is important to mention that the aforementioned effect of the rs2227956 variant seems to be opposite in multiple sclerosis where the C allele was associated with the risk of disease.[21] In this scenario, the decrease in peptide-binding HSP70 specificity (because of the C allele) would impair its role in preventing the

accumulation of misfolded proteins and in inducing antiapoptotic mechanisms, thus being detrimental to neuron/glia survival.[18,21] Moreover, the rs2227956 variant was not associated with Graves' disease or with systemic lupus erythematosus.[13,22] Therefore, the putative role of the variant on autoimmunity calls for further research as it seems that the consequence of its molecular effect might differ according to the affected tissue. Nevertheless, the protective effect of the rs2227956 C allele against vitiligo was further confirmed through haplotype analysis being GAC, the only major haplotype bearing the rs2227956 C allele, the one which was related to the protective effect (Table 3), a finding that was similar to the report of Toumi et al. in pemphigus foliaceus,[9] hinting a probable association with skin-specific autoimmune diseases.

Regarding HSP70 gene variants with the clinical characteristics of vitiligo patients, we did not find any associations. This result seems to contrast with previous research which found a correlation of high HSP70 expression with disease activity[17] but also remarks that the role of the protein is not necessarily related to its genetic variants. Both the latter and the lack of association with clinical characteristics have been observed by our group in previous work regarding other molecules/gene variants in relation to vitiligo.[23,24] Nevertheless, we cannot rule out whether HSP70 gene variants could be related to vitiligo characteristics in other populations or to features not considered in here such as the vitiligo area score index.

The present study represents the first approach assessing HSP70 genetic variants participation in vitiligo. Unfortunately, one of its limitations was the unavailability to measure HSP70 levels/expression which would have been useful to have a wider picture of the effect of the studied SNVs. In this sense, we hope our findings could be replicated in other populations hopefully overcoming the latter and other limitations, so to clarify the role of HSP70 genes in vitiligo susceptibility.

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