Original paper

Association of the HLA-G rs66554220 variant with non-segmental vitiligo and its clinical features in Northwestern Mexico population

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Abstract

Introduction: The HLA-G molecule functions as a critical immunomodulatory checkpoint, its expression is significantly associated with pathological processes that may be responsible in part for autoimmune conditions such as non-segmental vitiligo (NS-V), characterized by chronic skin depigmentation. In this sense, the rs66554220 (14 bp ID) variant located in the 3'UTR, implicated in the regulation of HLA-G production, is associated with autoimmune diseases.
Aim: To evaluate the role of the HLA-G rs66554220 variant in NS-V and its clinical features in Northwestern Mexicans.
Material and methods: We genotyped the rs66554220 variant by SSP-PCR in 197 NS-V patients and 198 age-sex matched non-related healthy individuals (HI).
Results: Del allele and genotype Del/Ins were the most prevalent in both study groups (NS-V/HI = 56%/55% and 46.70%/46.46%, respectively). Despite lacking association between the variant and NS-V, we found an association of the Ins allele in different inheritance models with familial clustering, onset of the illness, universal clinical subtype and Koebner's phenomenon.
Conclusions: The rs66554220 (14 bp ID) variant is not a risk factor for NS-V in the Mexican population studied. To our knowledge, this is the first report about the topic in the Mexican population and worldwide that includes clinical features related with this HLA-G genetic variant.

Key words: vitiligo, Mexican population, HLA-G 14 bp, rs66554220, Del/Ins.

Introduction

Non-segmental vitiligo (NS-V) is the most common skin acquired and chronic depigmentation disorder [1]. Its pathophysiology results from the interrelation between immunological and non-immunological factors and has recently drawn attention in the scientific community [2]. NS-V has an autoimmune nature derived from multifactorial aetiology with chronicity, variable treatment outcome, relapse of macules and decline in the quality of life in which the innate and adaptive immunity interplay in their intracellular environment [3].

Many treatments have been proposed but recently, the ones involving immunological checkpoints have taken the central stage particularly by their function in immunotolerance [1]. HLA-G, a non-classical MHC-Ib molecule, could be a new target in NS-V because of its immunomodulatory function as a checkpoint related with autoimmunity consequent from a consequence of uncontrolled activation of cellular effectors [4]. Moreover, it has been associated with autoimmune diseases such as systemic lupus erythematosus [5], multiple sclerosis [6], and psoriasis [7]. In this context, it might also be related

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to vitiligo since it is reported that 30% of vitiligo patients and their first-degree relatives have ≥ 1 autoimmune disease compared with the general population [8].

The HLA-G molecule, named as the "shield against immune aggression", is encoded by the HLA-G gene located on chromosome 6p22.1 [9]. Exogenous factors regulate its expression transcriptionally and post-transcriptionally directly by the union in its three prime untranslated region (3′-UTR) [9]. According to some reports [4, 9–12], the rs66554220 is the most studied single nucleotide variant (SNV) in this genic region, which produces a 14 bp Insertion/Deletion (Indel, ID) fragment related with increased expression and increased levels of sHLA-G [13, 14], which in turn has been previously associated with autoimmune disorders [6, 15] including vitiligo [10]. In the Mexican population from Jalisco with diabetes mellitus 2, the Del allele (minor allele) is the most prevalent [16]. Nevertheless, there no reports of the association with NS-V in the Mexican population.

Aim

To evaluate the role of the HLA-G rs66554220 variant in non-segmental vitiligo (NS-V) and its clinical features in Northwestern Mexicans.

Material and methods

This is a sex-age matched case-control study approved by the Biomedical Research Ethics Committee of the General Hospital of Culiacan, Sinaloa, Mexico where individuals were recruited, and it was performed with the collaboration of the Immunology Laboratory in Universidad de Guadalajara. All participants signed written informed consent and were at least 3 generations ascendant in Northwestern Mexico (Sonora, Sinaloa, Durango and Nayarit).

The characteristics of the individuals included in this report are mentioned previously in Ochoa-Ramírez *et al.* [17]. The control group was composed by 198 healthy individuals (HI) aged 41 ±18 years, and the study group included 197 individuals aged 42 ±17 years, in whom the clinical diagnosis of vitiligo was confirmed by the dermatologist. For the latter group, information about the following clinical features were obtained: onset, classified as early (< 20 years old) and late (≥ 20 years old) based on the reports that up to 50% of patients develop NS-V before the age of 20 years old [18, 19]; initial macule site and trigger (the variable stress was referred by the patients without psychological assessment), familial clustering, concomitant disorders, disease activity, established as stable if no lesions had appeared/extended in a period ≥ 1 year; VIDA score, Koebner's phenomenon, and disease duration.

Genetic analysis

DNA from participants was isolated from peripheral blood samples using the method of Gustincich *et al.* [20]. Genotyping for the rs66554220 (14 bp ID) variant was performed by Single Specific Primer-Polymerase Chain Reaction (SSP-PCR) using primer sequences modified from García-González *et al.* [16], to which two nucleotides were added at the beginning of each primer in order to adjust the two primers to the same alignment temperature, as follows: F: 5'-GTG ATG GGC TGT TTA AAG TGT CAC C-3' and R: 5'-GGA AGG AAT GCA GTT CAG CAT GA-3'. The SSP-PCR reactions were performed using 20 ng of genomic DNA in a total volume of 10 µl, containing 1X PCR buffer, 1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.3 mM of each primer and 0.25 U of recombinant Taq DNA polymerase. All reagents used in SSP-PCR including primers were obtained from Invitrogen (Life Technologies Corporation, Carlsbad, CA, USA). Later, the reaction was carried out in a thermal cycler Aeris (Esco® Lifesciences Group, Changi, Singapore) with the following conditions: initial denaturation at 94°C for 4 min, followed by 30 cycles of 26 s each at 94°C, 65°C and 72°C, and final extension at 72°C for 7 min. Fragments of 210 pb (Deletion) or 224 pb (Insertion) were obtained. These fragments were visualized in a 6% polyacrylamide (Golden Bell Reactivos, Jalisco, MX) (29 : 1) gel electrophoresis in an OWL P9DS camera (Thermo Fisher Scientific, Waltham, MA USA) stained with silver nitrate (Golden Bell Reactivos, Jalisco, MX). As a quality control, 10% of the genotypes was repeated by a second blind analyst.

Statistical analysis

Hardy-Weinberg Equilibrium (HWE) and χ^2 of alleles and genotypes were calculated using DeFinetti software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Genetic association tests and associations of clinical variables were evaluated also by sex-age matched conditional regression and binary logistic regression, respectively, in IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, N.Y., USA). A p-value < 0.05 and 95% CI were considered significant.

Results

Description of clinical features

Most of the patients with NS-V presented initial macules in superior and inferior distal extremities mostly of late onset appearance and, as mentioned in Table 1, 22% of them referred different causes as triggering (injury, sun exposure and other diseases). Also, over half of the NS-V patients reported familial clustering. Similarly, 49.74% presented concomitant disorders recognised as autoimmune (10.83% of these with familial clustering), thyroid, allergic, metabolic and other. Among the in-

Table 1. Clinical data of vitiligo patients

Parameter	*N* (%)
Stress as triggering	117 (78)
Familial clustering	95 (53.07)
Presence of autoimmune disorders:	32 (16.50)
Hashimoto's disease	18 (9.28)
Graves' disease	5 (2.58)
Psoriasis	4 (2.06)
Arthritis	2 (1.03)
Other*	3 (1.55)
With familial clustering and autoimmune disorder	21 (10.83)
Initial macule time:	
Late onset (> 20 y)	111 (57.51)
Early onset (< 20 y)	82 (42.49)
Clinical subtype:	
Focal	12 (6.19)
Acrofacial	18 (9.28)
Generalized	150 (77.32)
Universal	14 (7.21)
With Koebner's phenomenon	46 (24.47)
VIDA score:	
–1	11 (5.67)
0	66 (34.02)
1	57 (29.38)
2	22 (11.34)
3	30 (15.46)
4	8 (4.13)

*VIDA score meaning: -1, stable at least 1 year and spontaneous repigmentation; 0, stable for at least 1 year; +1, active in past 1 year; +2, active in past 6 months; +3, active in past 3 months; +4, active in past 6 weeks. *Included: lichen planus, ankylosing spondylitis, thrombocytopenic purpura.*

herent characteristics of NS-V, the most prevalent was the disseminated clinical subtype (generalized + universal = 84.53%). Moreover, more than 60% of the patients presented with active disease, of which 19.59% suffered from fast depigmentation within 6 weeks to 3 months (VIDA score 4 and 3, respectively) prone to a generalized-universal depigmentation phenotype related with a worse outcome. In relation with activity, 24.5% present with Koebner's phenomenon and, finally, the average duration of the disease was 16.03 ±14.05 years.

Genetic association study

Genotypic frequencies of the rs66554220 (14 bp ID) variant in both study groups agreed HWE, $p > 0.05$. As shown in Table 2, the Del allele and genotype Del/Ins were the most prevalent in both study groups. Nonetheless, no statistically significant differences were observed in a general way and after sex-age adjustment. On the other hand, we found association as a risk factor when we compared some of the different inheritance models with the following clinical characteristics (Table 3): 1) when comparing Ins allele carriers with first-degree familial clustering vs no familial clustering ($p = 0.037$) and vs. second-degree as baseline (Ins/Ins: $p = 0.02$, OR = 10.50, 95% CI: 1.08–102.49, data not shown) and Koebner's phenomenon ($p = 0.04$). 2) When we compared the Del/Del + Del/Ins genotype in the recessive model with the different clinical subtypes, it associates with the acrofacial ($p = 0.03$), generalized ($p = 0.006$) and universal ($p = 0.03$) subtypes using the focal subtype as baseline.

In addition, we observed an association as a protective factor on the following inheritance models: 1) Ins allele with early onset ($p = 0.02$); 2) Del/Ins in the codominant and Del/Ins + Ins/Ins dominant carrier models with stress as the triggering of the disease ($p = 0.004$ and $p = 0.007$, respectively); 3) Ins allele and Ins/Ins genotype with the different clinical subtypes (focal subtype as reference, $p < 0.04$), and Del/Ins with acrofacial, ($p = 0.03$); and 4) the dominant model of Del/Ins + Ins/Ins carriers with acrofacial ($p = 0.004$) and generalized ($p = 0.02$).

The feature's presence of autoimmune disease, and disease activity were not found to be associated with the rs66554220 (14 bp ID) SNV (data not shown).

Discussion

HLA-G shows expression in autoimmune skin disorders [21] and could be present in NS-V lesions. The SNVs in their gene associated with the level of in situ expression of the molecule, could contribute to the risk of developing NS-V, recalling their role in the homeostasis, pigmentation, and regulation of the immune response in the skin [22].

Herein, we propose the HLA-G rs66554220 variant as a biomarker in the course-prognostic of clinical features of vitiligo in Northwestern Mexican population as it had been proposed in other diseases with discordant results [23–25]. Nevertheless, the first study association about the rs66554220 variant was in Koreans, where the Ins/Ins genotype in a recessive model seems to be a risk factor for NS-V [10]. On the other hand, even though the aforementioned study is the only study that supports our findings, Veiga-Castelli *et al.* [26] studied SNVs in the *HLA-G* gene and NS-V patients and reported the prevalence of the Del allele in healthy individuals, however, did not analyse the possible genetic association of the rs66554220 variant with the clinical features of NS-V in Brazilians.

The HLA-G expression can be upregulated by interferons, interleukin-10 and other factors such as miRNAs under certain pathological conditions prompting anti-inflammatory and Th2-response [27]. Primordially, it is recognized as a tolerogenic molecule [28], normally absent

Table 2. Allelic and genotypic frequencies of the rs66554220 variant (14 bp ID) and association study according to inheritance models

Inheritance models	Healthy individuals *n* = 198 (%)	Vitiligo patients *n* = 197 (%)	(χ^2) *p*-value	OR [95% CI]
Allele:				
Del	109 (55)	111 (56)	Reference	
Ins	89 (45)	86 (44)	(0.05) 0.82	0.97 [0.73–1.28]
Co-dominant model:				
Del/Del	63 (31.82)	64 (32.49)	Reference	
Del/Ins	92 (46.46)	92 (46.70)	(0.00) 0.95	0.98 [0.63–1.55]
Ins/Ins	43 (21.72)	41 (20.81)	(0.05) 0.82	0.94 [0.54–1.63]
Dominant model:				
Del/Del	63 (31.82)	64 (32.49)	Reference	
Del/Ins + Ins/Ins	135 (68.18)	133 (67.51)	(0.02) 0.89	0.97 [0.64–1.48]
Recessive model:				
Del/Del + Del/Ins	155 (78.28)	156 (79.19)	Reference	
Ins/Ins	43 (21.72)	41 (20.81)	(0.05) 0.83	1.06 [0.65–1.71]

OR – odds ratio, χ^2 – value of χ^2, p-value – significance defined by χ^2 test; 95% CI – confidence interval of 95%.

on healthy tissues except for trophoblast, cornea, and thymus [29, 30]. In autoimmune disorders, the expression of this molecule may be responsible in part for the regulation of the autoimmune effector $CD4^+$ and $CD8^+$ T lymphocytes, NK cells, monocytes, and dendritic cells (DCs), underlying the pathogenesis of these disorders, creating an immune-suppressive milieu [28]. NS-V is not the exception, even though HLA-G is not normally expressed in the skin immune system [30], ectopic HLA-G expression and derived from exosomes has been described in other skin pathologies [29, 31].

Moreover, it is well known that HLA-G production, mRNA stability *in vivo* and protein production are intrinsically regulated by the 3'UTR of the HLA-G gene which can be affected by the rs66554220 single nucleotide variant (14 bp ID) genotype, for example, the Ins/Ins genotype, overall, is associated with lower HLA-G production compared to the Del/Ins and Del/Del genotype [31]. In our population, the Del/Ins genotype was the most common in both groups (HI = 46.46% and NS-V = 46.70%), which is concordant with a previous report on Western Mexicans (Del/Ins = 53%) [16] and Iranians (Del/Ins = 64%) [32], however, in other populations such as Indians [33], the Del/Del genotype is the most prevalent. Unfortunately, other publications only mention allelic frequencies of this SNV [28, 34].

In this sense, the distribution of the Del allele identified as the most prevalent in our population matches reports on Western Mexicans [16], Asians [10, 32–34] and South Americans [26]. Based on their ascendance background, it is reported that the Del allele is prevalent in up to 60% in Africans, Europeans and East Asians [26] including haplotype analyses [13, 14], except in Saudi Arabians [35], Egyptians [23], Iraqis [24] and Tunisians [25]. The genetic substructuring could explain the balanced selection worldwide, especially in genes with the important immunological function such as HLA-G.

Although our results seem to point to a lack of association of the HLA-G rs66554220 SNV with NS-V risk, we did observe some associations when analysing its clinical characteristics. It is important to mention that previous studies did not explore this topic. In this regard, we propose the Ins allele as a modifier genetic factor in different inheritance models in clinical features of NS-V such as family clustering in the first grade, onset of the disease, Koebner's phenomenon and depigmentation progress. All these associations have not been previously reported in the literature. In other diseases like Behcet's disease, non-Hodgkin lymphoma, and systemic lupus erythematosus, the Del/Ins genotype has been associated as a risk factor in outcome of clinical features such as concomitant autoimmune disorders [25], poor prognosis [23] and skin manifestations [15], respectively. Our results suggest that different HLA-G variant profiles could be associated with different clinical expressions of the disease, but also that the differences between ethnic groups are important in this kind of studies. Even though meta-analysis discards the association of this variant with the susceptibility to overall autoimmune disease [11], it is associated with susceptibility to a subgroup of autoimmune diseases that includes systemic lupus erythematosus [12], which suggests common etiopathogenic mechanisms among these disorders and their identification can help develop new therapeutic approaches [36], as biomarkers

Table 3. Association of the rs66554220 variant (14 bp ID) genotype with non-segmental vitiligo features

Clinical features	Inheritance models								
	Allele		Co-dominant			Dominant		Recessive	
	Del *n* (%)	Ins *n* (%)	Del/Del *n* (%)	Del/Ins *n* (%)	Ins/Ins *n* (%)	Del/Del *n* (%)	Del/Ins+Ins/Ins *n* (%)	Del/Del+Del/Ins *n* (%)	Ins/Ins *n* (%)
Triggering:									
Other[1]	15 (44)	18 (56)	4 (12.12)	21 (63.64)	8 (24.24)	4 (12.12)	29 (87.88)	25 (75.76)	8 (24.24)
Stress	67 (57)	50 (43)	43 (36.75)	47 (40.17)	27(23.08)	43 (36.75)	74 (63.25)	90 (76.92)	27 (23.08)
(χ^2) *p*-value	Ref	n.s.	Ref	(8.18) 0.004	n.s.	Ref	(7.26) 0.007	Ref	n.s.
OR				0.21			0.24		
[95% CI]				[0.07–0.66]			[0.08–0.72]		
Familial clustering:									
No	50 (59)	34 (41)	32 (38.09)	35 (41.67)	17 (20.24)	32 (38.09)	52 (61.91)	67 (79.76)	17 (20.24)
First grade	14 (44)	18 (56)	8 (25)	12 (37.50)	12 (37.50)	8 (25)	24 (75)	20 (62.50)	12 (37.50)
(χ^2) *p*-value	Ref	(4.31) 0.037	Ref	n.s.	n.s.	Ref	n.s.	n.s.	n.s.
OR		1.85							
[95% CI]		[1.03–3.30]							
Initial macule time:									
Late onset (> 20 y)	57 (51)	54 (49)	35 (31.53)	44 (39.64)	32 (28.83)	35 (31.53)	76 (68.47)	79 (71.17)	32 (28.83)
Early onset (< 20 y)	52 (63)	30 (37)	29 (35.37)	45 (54.88)	8 (9.75)	29 (35.37)	53 (64.63)	74 (90.25)	8 (9.75)
(χ^2) *p*-value	Ref	(5.03) 0.02	Ref	n.s.	(6.88) 0.008	Ref	n.s.	Ref	n.s.
OR		0.63			0.30				
[95% CI]		[0.41–0.94]			[0.12–0.75]				
Koebner's phenomenon:									
No	84 (59)	58 (41)	52 (36.62)	63 (44.37)	27 (19.01)	52 (36.62)	90 (63.38)	115 (80.99)	27 (19.01)
Yes	22 (47)	24 (53)	10 (21.74)	23 (50)	13 (28.26)	10 (21.74)	36 (78.26)	33 (71.74)	13 (28.26)
(χ^2) *p*-value	Ref	(4.10) 0.04	Ref	n.s.	n.s.	Ref	n.s.	Ref	n.s.
OR		1.63							
[95% CI]		[1.01–2.61]							
Clinical subtype:									
Focal	7 (30)	15 (70)	2 (9.10)	9 (40.90)	4 (50)	2 (9.10)	20 (90.9)	11 (50)	11 (50)
Acrofacial[2]	12 (67)	6 (33)	9 (50)	6 (33.33)	3 (16.67)	9 (50)	9 (50)	15 (83.33)	3 (16.67)
Generalized[3]	83 (55)	67 (45)	50 (33.33)	66 (44)	34 (22.67)	50 (33.33)	100 (66.67)	116 (77.33)	34 (22.67)

Table 3. Cont.

Clinical features	Inheritance models								
	Allele		Co-dominant			Dominant		Recessive	
	Del *n* (%)	Ins *n* (%)	Del/Del *n* (%)	Del/Ins *n* (%)	Ins/Ins *n* (%)	Del/Del *n* (%)	Del/Ins+Ins/Ins *n* (%)	Del/Del+Del/Ins *n* (%)	Ins/Ins *n* (%)
Universal[4]	8 (54)	6 (46)	3 (21.42)	9 (64.29)	2 (14.29)	3 (21.42)	11 (78.58)	12 (85.71)	2 (14.29)
(χ^2) *p*-value	Ref	(10.98) 0.001[2]	Ref	(4.55) 0.03[2]	(9) 0.003[2]	Ref	(8.31) 0.004[2]	Ref	(4.84) 0.03[2]
		(10.22) 0.001[3]			(8.82) 0.002[3]		(5.35) 0.02[3]		(7.42) 0.006[3]
		(4.16) 0.04[4]							(4.73) 0.03[4]
OR		0.21[2]		0.15[2]	0.06[2]		0.10[2]		5[2]
		0.34[3]			0.12[3]		0.20[3]		3.41[3]
		0.36[4]							6[4]
[95% CI]		[0.08–0.54][2]		[0.02–0.94][2]	[0.01–0.45][2]		[0.02–0.56][2]		[1.12–22.30][2]
		[0.17–0.67][3]			[0.03–0.59][3]		[0.05–0.89][3]		[1.36–8.55][3]
		[0.14–0.97][4]							[1.08–33.32][4]

[1]Included: injury, sun exposure and other diseases; in the clinical subtype, comparisons the significance are indicated in uppercase: 2 for focal vs acrofacial, 3 for focal vs generalized and 4 for focal vs. universal; χ^2 – chi-square value; p-value – significance defined by χ^2 test, OR – odds ratio, 95% CI – confidence interval of 95%, Ref – reference of baseline in comparisons made, n.s. – non-significant.

mostly used to understand how genetic variants are associated with levels of molecules, to aid in the stratification of clinic prognosis, and to identify new therapeutic targets [37].

Conclusions

The rs66554220 SNV (14 bp ID) is not a risk factor for NS-V in the Mexican population but appears to relate to its course prognosis. Nevertheless, further functional studies are needed since the differential expression of HLA-G may be beneficial or harmful depending on the underlying condition and ethnic group [38]. HLA-G may help in defining novel strategies to control the immune response against the underlying disorder given that different evolutionary lineages are conservative in 3'UTR, introns, and 5'UTR of the HLA-G gene [39] also in strong linkage disequilibrium with HLA-II [40]. This is the first report about the topic on the Mexican population and worldwide which includes clinical features related with the genetic variant.

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Conflict of interest

The authors declare no conflict of interest.

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