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Association of human beta-defensin 1 gene polymorphisms with nonsegmental vitiligo

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Summary

Background. Vitiligo is a pigmentation disorder of autoimmune aetiology. Polymorphisms in beta-defensin genes have been linked to a predisposition to some autoimmune disorders.

Aim. To evaluate the role of polymorphisms in *DEFB1*, the gene encoding for human beta-defensin (HBD)-1 and its 5′ untranslated region in nonsegmental vitiligo.

Methods. In total, 354 participants [171 patients with non-segmental vitiligo and 183 age and sex-matched healthy controls (HCs)], were genotyped by the PCR-restriction fragment length polymorphism (RFLP) method. For 80 of these individuals (40 patients and −40 HCs) serum HBD-1 was also measured by ELISA.

Results. The -44 G allele, CG genotype and GGG haplotype increased the risk for vitiligo ($P < 0.02$ in all cases), whereas the -20 AA genotype seems to be protective ($P = 0.04$). Serum HBD-1 levels were lower in patients with vitiligo than in HCs ($P < 0.01$), as well as in patients with active vitiligo compared with those with stable vitiligo and with HCs ($P < 0.05$ in both cases),

Conclusion. Our results suggest that *HBD-1* and its gene polymorphisms may modulate vitiligo susceptibility and/or disease activity. This is the first report, to our knowledge, of the association of serum HBD-1 levels and *DEFB1* gene polymorphisms with vitiligo.

Introduction

Vitiligo is an acquired pigmentation disorder characterized by the loss of epidermal melanocytes and/or their function. It is a relatively common disorder, with a prevalence rate of 0.2–1%.[1] Although the exact aetiology of vitiligo remains elusive, autoimmunity is believed to play an important role in disease pathogenesis, as vitiligo is often associated with autoimmune diseases.[2,3] There is also evidence for a direct role for interferon-γ-producing CD8+ cytotoxic T lymphocytes (CTLs) in the progression of vitiligo, corresponding to a T helper (Th)1 response.[4,5] In addition, Th17 response has already been acknowledged to play an important role in vitiligo pathogenesis.[5–7]

Th17 response is characterized by the elicitation of antimicrobial peptides (AMPs) through interleukin (IL)-17A, IL17F and IL-22 signalling, leading to localized inflammation.[8] An excess of constitutively expressed AMPs such as human beta-defensin (HBD)-1 may also contribute to local inflammation; for HBD-1,

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this is due to its ability to chemoattract neutrophils, immature dendritic cells and T cells directly through chemokine receptor (CCR)6 signalling and indirectly by HBD-3 induction.[9]

Certain polymorphisms in *DEFB1*, the gene encoding for HBD-1, may affect its transcription rate and the expression of HBD-1 protein.[10,11] In particular, polymorphisms in the 5′ untranslated region (UTR) of *DEFB1* exert this effect by altering a putative transcription factor binding site for the nuclear factor-κB p105 subunit.[12]

Considering autoimmunity, HBD-1 and its gene polymorphisms have been evaluated in psoriasis (PSO), type 1 diabetes (T1D), oral lichen planus (OLP), inflammatory bowel disease (IBD), and systemic and cutaneous lupus erythematosus (SLE and CLE, respectively), with variable degrees of association identified.[11,13–18] However, to date, there are no similar studies regarding vitiligo susceptibility.

Considering the role of HBD-1 and its gene 5′UTR polymorphisms in autoimmune disease, we hypothesized that they may be involved in vitiligo pathogenesis and/or its clinical characteristics in a Mexican population.

Methods

The study was approved by the Biomedical Research Ethics Committee of the General Hospital of Culiacan, Sinaloa, México, where it was performed. Written informed consent was obtained from all participants.

Subjects

The study group consisted of 171 patients with nonsegmental vitiligo (80 males, 91 females) who were referred to General Hospital of Culiacan, Sinaloa, Mexico. Vitiligo diagnosis was made by dermatologists based on clinical findings according to the classification of the Vitiligo Global Issues Consensus Conference, Bordeaux, 2011.[19] A questionnaire was prepared in order to identify relevant aspects such as the age of onset, anatomy of the lesions, disease activity, concurrent diseases and family background of vitiligo. For disease activity, disease was considered to be stable when repigmentation occurred or no new lesions had appeared in a time period of ≥ 1 year.

The healthy control (HC) group consisted of 183 sex-age matched individuals (90 males and 93 females) who had no vitiligo or any autoimmune diseases and did not have a family background of vitiligo.

Genetic analyses

Peripheral blood samples from participants were collected in tubes with EDTA anticoagulant, and DNA was isolated from the samples. Genotyping for the -52G/A (rs1799946), −44C/G (rs1800972) and -20G/A (rs11362) polymorphisms was performed following the protocol of Estrada-Aguirre *et al.*[20] A percentage (5%) of the genotypes was confirmed by sequencing of PCR products (Macrogen Inc., Seoul, Korea).

Serum analyses

Serum samples were separated from coagulated blood and stored at −70°C until use. HBD-1 levels were assessed in serum samples from 80 of the 354 participants (40 patients and 40 HCs; age range 18–45 years) by ELISA. All samples were analysed at the same time using an ELISA kit (HBD-1 ELISA Kit LS-F549; LifeSpan Biosciences Inc., Seattle, WA, USA) according to the manufacturer's instructions.

Statistical analyses

Hardy–Weinberg equilibrium (HWE) for the polymorphisms was calculated with the use of deFinetti software (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Haplotypes were constructed and analysed using SNPStats software (http://bioinfo.iconcologia.net/SNPstats).[21] The remaining analyses were performed with SPSS Statistics (v20; IBM SPSS, Armonk, NY, USA). Briefly, conditional logistic regression was used to obtain age-sex adjusted ORs and 95% CIs for case–control comparisons. Associations of clinical variables with polymorphisms were evaluated by logistic regression. Group means differences in serum HBD-1 levels were analysed by Mann–Whitney *U*-test. Graphics were performed with GraphPad Prism (v6; GraphPad, La Jolla, CA, USA). $P < 0.05$ was considered significant.

Results

Participant data

Clinical and demographical data of the 171 patients with nonsegmental vitiligo included in the present study are shown in Table 1. Genotype frequencies were in agreement with HWE in both the case and control groups.

Polymorphisms and vitiligo

Genotype frequencies were in agreement with HWE in both the case and control groups. As shown in

Table 2, both the -44 CG genotype and G allele frequencies were higher in patients with vitiligo, conferring a 1.77- and 1.83-fold increased risk of developing vitiligo, respectively ($P < 0.02$ for both), whereas the -20 AA genotype seems to be protective against vitiligo by > 45% ($P = 0.04$; OR = 0.53, 95% CI = 0.28–0.98). No statistically significant differences were observed between patients with vitiligo and HCs in allele and genotype frequencies of -52G/A. Furthermore, we found no association of -52G/A, -44C/G or -20G/A *DEFB1* polymorphisms with the clinical characteristics of vitiligo listed in Table 1 (onset, clinical type, disease activity, presence of autoimmune disease, Koebner phenomenon, sex and/or family history) (see Tables S1–S3).

Table 1 Clinical and demographical data of patients with vitiligo.

Characteristic	Total *n* = 171 (%)
Sex	
Male	80 (46.8)
Female	91 (53.2)
Onset	
Early (< 20 years old)	72 (42.1)
Late (≥ 20 years old)	99 (57.9)
Clinical type	
Focal†	8 (4.7)
Acrofacial	16 (9.3)
Vulgaris	133 (77.8)
Universalis	14 (8.2)
Disease activity	
Active (within 1 year)	98 (57.3)
Stable (≥ 1 year)	73 (42.7)
Family history of vitiligo*	97 (56.7)
Autoimmune disease	17 (9.9)
Koebner phenomenon	33 (19.3)

*Reported first-, second- and/or third-degree affected relatives; †bilateral lesions at one anatomical site (e.g. both eyelids).

Table 2 Allelic and genotypic frequencies of *DEFB1*–5′UTR polymorphisms in patients with vitiligo and healthy controls.

Genotype	wV (*n* = 171) *n* (%)	HCs* (*n* = 183) *n* (%)	*P*	OR (95% CI)†
-52 G/A (rs1799946)				
GG	81 (47.4)	89 (48.6)	–	Reference
GA	77 (45)	75 (41)	0.545	1.14 (0.75–1.74)
AA	13 (7.6)	19 (10.4)	0.411	0.71 (0.31–1.62)
A	0.30	0.31	0.806	1.05 (0.70–1.59)
-44 C/G (rs1800972)				
CC	82 (47.9)	111 (60.6)	–	Reference
CG	78 (45.6)	66 (36.1)	0.018	1.77 (1.10–2.85)
GG	11 (6.5)	6 (3.3)	0.103	2.35 (0.84–6.55)
G	0.29	0.21	0.010	1.83 (1.16–2.90)
-20 G/A (rs11362)				
GG	62 (36.2)	53 (29)	–	Reference
GA	81 (47.4)	87 (47.5)	0.271	0.76 (0.46–1.25)
AA	28 (16.4)	43 (23.5)	0.043	0.53 (0.28–0.98)
A	0.40	0.47	0.120	0.68 (0.42–1.10)

HCs, healthy controls; PwV, patients with vitiligo. *In agreement with Hardy–Weinberg equilibrium (-52G/A: $P = 0.59$, -44C/G: $P = 0.38$, -20G/A: $P = 0.53$); †adjusted OR and 95% CI for age and sex.

DEFB1 haplotypes and vitiligo

Linkage disequilibrium was observed between the three polymorphisms ($D' > 0.99$, r^2 -52G/A: -44C/G = 0.15, r^2 −52G/: -20G/A = 0.34, r^2 -44C/G: -20G/A = 0.26). As shown in Table 3, three major haplotypes were found, of which GGG was more frequent in patients with vitiligo, being associated with a 1.66-fold increased risk for developing the disorder ($P = 0.01$). Haplotypes were not associated with any of the aforementioned clinical characteristics of vitiligo (data not shown).

Human beta-defensin-1 and vitiligo

Analysis of serum HBD-1 levels showed a significant difference between patient and HC groups, with the patient group having lower HBD-1 levels ($P = 0.001$) (Fig. 1a). When categorized by disease activity, patients with active vitiligo were found to have decreased levels of HBD-1 compared with patients with stable vitiligo ($P = 0.02$) and HCs ($P < 0.001$), although there was no difference between the stable vitiligo and HC groups (Fig. 1b). Serum HBD-1 levels were not associated with any other clinical characteristic nor with any *DEFB1* genotype, allele or haplotype (see Table S4).

Discussion

There is currently little information regarding the role of HBD-1/*DEFB1* in vitiligo. In the present study, we evaluated the role of serum HBD-1 levels and three *DEFB1* 5′UTR polymorphisms in vitiligo, based on their previously reported association with other autoimmune diseases.[11,13–18]

We found that the G allele and CG genotype at position -44 and the GGG haplotype were associated with an increased risk of developing vitiligo (Table 2, Table 3). These findings are similar to previous reports in SLE, but different from reports on T1D, in which the allele and genotype appeared to have a protective effect.[15,17] These results could be attributable to an increase in the transcription rate of *DEFB1* mRNA

Table 3 Haplotype frequencies of *DEFB1*–5′ UTR polymorphisms in patients with vitiligo and healthy controls

Haplotype	PwV	HCs	*P*	OR (95% CI)
GCA	0.40	0.47	–	Reference
ACG	0.30	0.31	0.50	1.13 (0.8–1.59)
GGG	0.29	0.21	0.01	1.66 (1.13–2.45)
GCG	< 0.01	< 0.01	0.81	1.28 (0.17–9.34)

HCs, healthy controls; PwV, patients with vitiligo.

due to the presence of the -44 G allele, increasing constitutive HBD-1 production.[10–12] This could also be relevant for SLE and T1D, as observed previously.[15,17] Thus, our findings support the autoimmune hypothesis for vitiligo, considering the chemoattractant properties of HBD-1 for immature dendritic cells and memory T cells, which in the presence of 'danger' signals (e.g. oxidative stress, high levels of IL-6, IL-8 and heatshock protein 70) would promote initial autoantigen presentation and depigmentation flares, respectively,[5,9] a model similar to that previously suggested by Prado-Montes de Oca *et al.* for atopic dermatitis.[22]

Following age/sex adjustment, we found that the -20 AA genotype confers protection against vitiligo (Table 2), contrasting with previous observations for SLE, T1D and Crohn disease,[15,17,23]; but in agreement with a report on AD, which found the -20 GG genotype to be a genetic risk factor.[22] In this context, Zanin *et al.* mentioned that the -20 A allele appears to be related to lower both HBD-1 and gene expression at the tissue level,[23] which might also explain its role as a risk factor for human immunodeficiency virus infection.[20] Nevertheless, recent research has failed to prove such an effect on HBD-1 levels, meaning that the *DEFB1* -20G/A polymorphism needs to be investigated further regarding its functional role.[11]

In contrast to the protective effect of the -52 A allele and the risk increase of the GCA haplotype observed for SLE,[17] no association of -52G/A and the remaining haplotypes (GCA, ACG and GCG) with vitiligo was found in our study. This difference might be related to the fact that despite both diseases being autoimmune and having cutaneous manifestations, they are driven by distinct immunological components: vitiligo by CD8+ CTLs and SLE by antibodies.[5,17] Nevertheless, we cannot discard the genetic differences between Mexican and Brazilian populations either, as both explanations could be pertinent to the rest of the aforementioned findings.

Regarding HBD-1 levels, they were unexpectedly lower in patients with vitiligo than in HCs (Fig. 1a), being similar to those for T1D but opposite to those

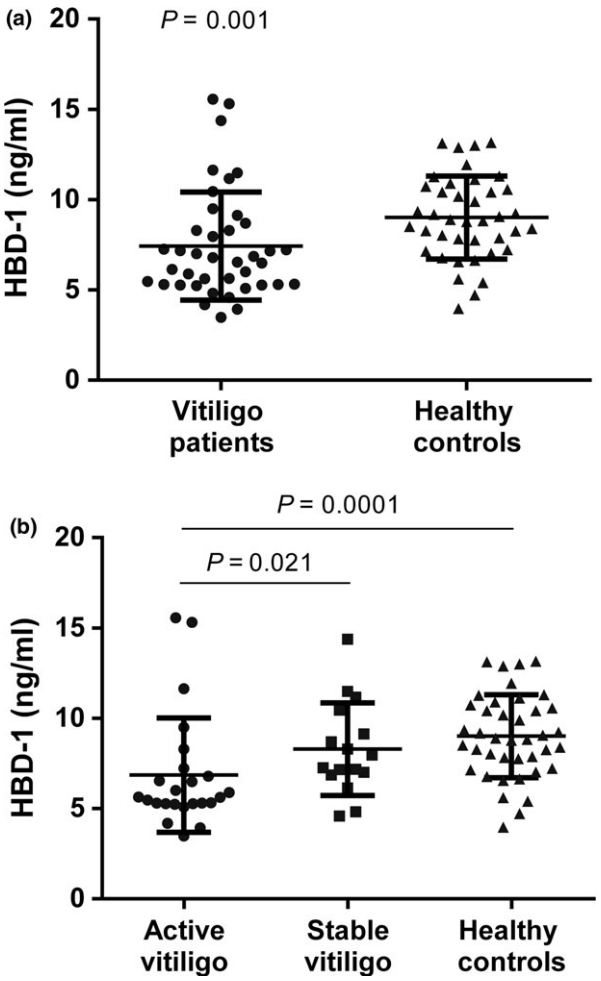

Figure 1 (a,b) Serum human beta-defensin (HBD)-1 levels in patients with vitiligo and in healthy controls (HCs). (a) HBD-1 levels of 40 patients with nonsegmental vitiligo vs. 40 HCs. (b) The patient group was subdivided according to disease activity into active (n = 24) and stable (n = 16) vitiligo, and their HBD-1 levels were compared against each other and against the HC group. HBD-1 levels were assessed by ELISA, values are expressed as the mean ± SD.

for OLP, CLE and PSO.[11,14,18] These relationships might be due to the similar aetiology of vitiligo and T1D, as both are mediated by CD8+ CTLs, in contrast to OLP, CLE and PSO, which involve chronic inflammation.[11,14,18] However, the methodological approach for HBD-1 assessment may also be responsible; the previous study in T1D was performed on serum (as in our study), whereas the OLP study used saliva and the CLE and PSO studies used skin biopsies.[11,14,18] Indeed, this could help to explain the lack of effect of *DEFB1* polymorphisms on systemic HBD-1 levels in our study, which contrasts with the effect observed in analyses of local

gene or protein expression.[10,11,18] Therefore, it is necessary to perform studies linking local and systemic HBD-1 expression in order to better explain the functional role of its gene polymorphisms, particularly in vitiligo.

Interestingly, we found that patients with active vitiligo had lower HBD-1 levels than those with stable vitiligo, but no differences were observed between stable cases and HCs (Fig. 1b), thus suggesting that low systemic HBD-1 levels are related to disease activity. A possible explanation for this result is that the excessive CD8+ CTL subpopulation activation, characteristic of active vitiligo, demands high glucose expenditure, negatively affecting HBD-1 production, as it is also glucose-dependent.[4,14,24,25] It is important to mention that we suggest this finding not as a causal effect but as a consequence of the disease, as we did not observe an association between serum HBD-1 levels and *DEFB1* gene polymorphisms, and the latter was not associated with disease activity or any other of the studied clinical characteristics of vitiligo (see Tables S1–S4).

Conclusion

It appears that *DEFB1* gene polymorphisms may modulate vitiligo risk whereas HBD-1 levels might be related to its pathogenesis. The present study is, to our knowledge, the first to associate HBD-1/*DEFB1* with vitiligo. Further genetic and/or functional studies on this defensin are required in order to define its exact role in vitiligo susceptibility and/or pathogenesis.

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What's already known about this topic?

- Vitiligo risk and pathogenesis have been observed to be influenced by immunogenetic factors.
- Polymorphisms in HBD-1 and its gene *DEFB1* have been associated with autoimmune diseases.

What does this study add?

- The -44 G allele, CG genotype and GGG haplotype increased the risk for vitiligo, whereas the -20 AA genotype seemed to be protective.
- Serum HBD-1 levels were lower in patients with vitiligo than in HCs.
- They were also lower in patients with active than those with stable vitiligo.
- This is the first study to propose a possible association between the *DEFB1* -44 (rs1800972) G allele and vitiligo risk, and between HBD-1 levels and disease activity.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Allelic and genotypic frequencies of *DEFB1* 5′UTR polymorphisms in patients with vitiligo according to sex and family history of vitiligo.

Table S2. Allelic and genotypic frequencies of *DEFB1* 5′UTR polymorphisms in patients with vitiligo according to disease activity, clinical type, and type of onset.

Table S3. Allelic and genotypic frequencies of *DEFB1* 5′UTR polymorphisms in patients with vitiligo according to positivity to autoimmune comorbidity and Koebner phenomenon.

Table S4. Human beta-defensin 1 (HBD1) levels according to the demographic and clinical characteristics of vitiligo.

