

Association of β -Defensin 1 Single Nucleotide Polymorphisms with Atopic Dermatitis

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Key Words

Atopic dermatitis • *DEFB1* gene • Haplotype inference • Single nucleotide polymorphisms

Abstract

Background: Atopic dermatitis (AD) is a chronic multifactorial allergic disease with unclear etiology. The antimicrobial human β -defensin 1 is chemotactic for dendritic cells, which are important regulators of allergic immune responses. In an attempt to identify useful markers that could predict susceptibility to AD, we investigated single nucleotide polymorphisms (SNPs) of the β -defensin 1 gene (*DEFB1*) with potential functional consequences. **Methods:** Four SNPs of the *DEFB1* gene were genotyped either by real-time polymerase chain reaction or polymerase chain reaction-restriction fragment length polymorphisms in 59 patients with AD and 151 controls from the Mexican population. Correlation analyses were carried out between genetic, environmental and clinical variables in AD patients. **Results:** The genotypes associated with susceptibility to AD and no other allergy were 692

GG (OR = 3.21, 95% CI 1.37–7.34) and 1654 AA (OR = 17.37, 95% CI 1.62–860.83). The allele 668 C is a risk factor for AD (OR = 2.23, 95% CI 1.22–4.01) and the allele A in site 1836 correlates with earlier age at onset (Spearman's ρ = 0.232; p = 0.03). The prolonged duration of breastfeeding correlates with earlier age at onset as well as with the severity of AD. **Conclusions:** The *DEFB1* gene is probably involved in the incidence and development of AD, but additional functional studies will be necessary to understand the biological role of these SNPs.

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Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease affecting 10–20% of children and 1–3% of adults worldwide [1]. The typical features of AD include pruritus, erythema, dry skin and inflammation, and it is associated with an increased frequency of both circulating and cutaneous allergen-specific Th2-

like cells secreting interleukin (IL)-4 and IL-5 [2]. At least 20 genes have been implicated in AD, including filaggrin [3]. The majority of these overlap with other loci involved in immune diseases such as asthma and psoriasis. Thus, these genes are poor markers of isolated AD [4]. However, over 50% of patients with AD will develop asthma and approximately 75% will develop allergic rhinitis, evidence supporting the overlap in the molecular physiopathology proposed for these diseases [2]. Even so, this highlights the importance of searching for distinctive markers for each of these conditions.

Monocytes, mast cells, dendritic cells (DCs) and T cells are attracted by human β -defensins in vitro, highly conserved antimicrobial peptides that may play an important role in allergic diseases [5]. The β -defensins are considered as a link between the innate and adaptive immunity acting as 'microchemokines' [6]. Human β -defensin 1 (hBD-1) has not been previously studied in conjunction with AD, perhaps due to the fact that it is constitutively expressed in epithelia, neutrophils and keratinocytes. Nevertheless, its expression is upregulated both during gastritis produced by *Helicobacter pylori* and by IL-1 β [7] and interferon- γ in vitro [8]. Furthermore, there is growing evidence that hBD-1 plays a role in inflammatory processes [9–11], probably due to its chemotactic properties towards immature DCs [6], cells that define initiation and the type of the resulting immune response [12]. Interestingly, single nucleotide polymorphisms (SNPs) in *DEFB1* have been associated with asthma, another atopic disease with a similar Th2 response but different tissue affected [13, 14].

The *DEFB1* gene is found at the 8p23.2–p23.1 locus (MIM No. 602056) and is approximately 7.3 kb in size (SNP Browser 2.0, Applied Biosystems, Foster City, Calif., USA). Four SNPs in this gene have been studied, as their location in the gene suggests that they may induce functional alterations [15]. The SNPs analysed were: (1) *DEFB1* 668 C→G in exon 1 – a transversion that creates a putative site for nuclear factor- κ B binding, a nuclear factor involved in regulating the expression of proinflammatory cytokines and other defensins [16]; (2) *DEFB1* 692 A→G in exon 1 – adjacent to the sequence that encodes the signal peptide; (3) *DEFB1* 1654 G→A in exon 2 – adjacent to a Cys residue that forms one of three disulfide bridges, and therefore, can alter peptide stability; (4) *DEFB1* 1836 A→G in exon 2 – a putative polyadenylation site that may affect processes of transcription and/or translation [15].

Currently, there is little information available regarding the distribution of these *DEFB1* polymorphisms in

different populations [17–19]. Hence, we have designed polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLPs) for three of the aforementioned SNPs and genotyped them either using real-time (RT)-PCR or PCR-RFLPs. Due to technical difficulties, the remaining *DEFB1* 668 C→G was studied using RFLPs alone.

Material and Methods

Subjects

Samples of peripheral blood from patients diagnosed with AD (n = 59) were obtained at the Instituto Dermatológico de Jalisco in Guadalajara, Jalisco, Mexico. The age range of the subjects was 0.3–32 years and the male:female ratio was 1:0.96. All patients met the criteria of AD proposed by Hanifin and Rajka [20]. Control samples from unrelated individuals (n = 151, average age 28 $\pm$ 5 years, male:female ratio 1:0.98) without family or personal history of atopy were obtained from the Blood Bank of the Instituto Mexicano del Seguro Social (IMSS) at Centro Médico Nacional de Occidente (CIBO). Patients and controls were recruited during 2005. All of them were 'mestizo' from western Mexico; mestizos are defined as individuals that result from the admixture between Spaniards and Amerindians [21], who make up 80% of the total Mexican population [22]. The studied population is ethnically homogeneous according to Cerda-Flores et al. [23]. The control: patient ratio was 2.56:1, within the recommended limits for case-control studies [24]. The protocol was approved by the ethics committees both at our research center (CIBO-IMSS) and at the Instituto Dermatológico de Jalisco in Guadalajara. Samples of all participants were obtained after they had voluntarily signed an informed consent form. Additionally, a questionnaire was filled out by each patient which included the family history of allergies, dust exposure and information regarding several well-known factors that trigger AD. Severity was evaluated according to the eczema area and severity index [25], and for the correlation analysis, data were eliminated from those patients without relapse at the time of the interview.

PCR Amplification

PCR was performed in a reaction mixture that contained the following: 1.25 μ l of 10 $\times$ PCR buffer, 5 μ M of each primer, 0.05 mM of dNTPs, 2.12 mM of MgCl₂, 0.25 units of Taq polymerase (Invitrogen Inc., Carlsbad, Calif., USA), and 12.5 ng of DNA adjusted to 12.5 μ l with water. The primers used were those designed previously for RT-PCR typing [15], and the sizes of amplicons were 156 (the same for both *DEFB1* 668 C→G and *DEFB1* 692 A→G), 157 (*DEFB1* 1654 G→A) and 164 bp (*DEFB1* 1836 A→G). A single PCR program was standardized for all the reactions. This program consisted of an initial preheating to 50°C for 2 min, a denaturing step at 95°C for 10 min and 34 cycles of denaturing at 95°C for 15 s, annealing at 63°C for 1 min and extension at 72°C for 1 min. The PCR products were assessed by separating 0.3–0.5 μ g of the amplified products in a 6% (29:1) polyacrylamide gel for 120 min at 200 V, and they were visualized by silver staining.

Table 1. Genotype and allele frequencies of controls and AD patients with overlapping allergies

SNP	Controls			Patients with AD plus other allergies (n = 14)		
	genotype	frequency	allelic frequencies	genotype	frequency	allelic frequencies
<i>DEFB1</i> 668 C→G	CC	3 (2)	C = 0.195	CC	1 (7.1)	C = 0.25
	GC	53 (35.1)	G = 0.805	GC	5 (35.7)	G = 0.75
	GG	95 (62.9)		GG	8 (57.1)	
<i>DEFB1</i> 692 A→G	AA	24 (15.9)	A = 0.483	AA	0 (0)	A = 0.36
	AG	98 (64.9)	G = 0.517	AG	10 (71.4)	G = 0.64
	GG	29 (19.2)		GG	4 (28.6)	
<i>DEFB1</i> 1654 G→A	GG	0 (0)	G = 0.5	GG	0 (0)	G = 0.46
	AG	151 (100)	A = 0.5	AG	13 (92.9)	A = 0.54
	AA	0 (0)		AA	1 (7.1)	
<i>DEFB1</i> 1836 A→G	AA	145 (96)	A = 0.980	AA	11 (78.6)	A = 0.89
	AG	6 (4)	G = 0.020	AG	3 (21.4)	G = 0.11
	GG	0 (0)		GG	0 (0)	

Figures in parentheses are percentages. p values of the frequency comparison of alleles and genotypes of patients versus controls were not significant.

RFLPs Assay Design

Sequences of exon 1 (for the 668 and 692 sites) and exon 2 (for the 1654 and 1836 sites) of the *DEFB1* gene were obtained from GenBank (accession numbers U50930 and U50931). Genotyping assays were designed with GeneTool 2.0. software (BioTools, Inc., Edmonton, Canada), except for the 1654 site which was designed manually. The expected restriction fragments obtained with the enzymes utilized were as follows: for *DEFB1* 668 C→G, *BsmAI* [GTCTC(N)₁↓] 96 + 60 bp (CC), 156 bp (GG); for *DEFB1* 692 A→G, *DdeI* (C↓TNAG), 87 + 69 bp (GG), 69 + 44 + 43 bp (AA); for *DEFB1* 1654 G→A, *HpyCH4V* (TG↓CA), 152 + 5 bp (GG), 95 + 57 + 5 bp (AA); for *DEFB1* 1836 A→G, *RsaI* (GT↓AC), 88 + 76 bp (AA), 164 bp (GG). Where necessary, amplicons from human mitochondrial DNA not polymorphic for the recognition sequence were used as a positive control for digestion. The digestion products (3 µg) were loaded on a 12% polyacrylamide gel (29:1), electrophoresed for 120 min at 150 V and visualized by silver staining.

Real-Time PCR

TaqMan assays were performed in an ABI-PRISM 7000 Real-Time Thermocycler (Applied Biosystems). The reaction mixture contained 0.25 µl of primers with 6FAM™ MGB dye-labeled probe and VIC® MGB dye-labeled probe (40 × mixture), 5 µl of 2 × universal PCR Master Mix, 12.5 ng of DNA template, and water to a total volume of 11 µl. The sequences of the three primer sets have been reported elsewhere [15]. The thermocycling program included an initial preheating step to 50°C for 2 min, a denaturing step at 95°C for 10 min, and 50 cycles of denaturing at 92°C for 15 s and annealing/extension at 60°C for 1 min. The genotypes were assigned using the Sequence Detection System software, version 1.1 (Applied Biosystems), analyzing the thresh-

old cycle of amplification curves, and not only genotype assignment plots, and using at least two negative controls/probe/plaque. Random samples (12% of total sample size) were genotyped with both methods as a quality control for our results.

Statistical Analysis

Correlation analysis, allele and genotype frequencies were estimated with SPSS software version 10 (SPSS, Inc., Chicago, Ill., USA). Haplotype inference, linkage disequilibrium estimation and the Hardy-Weinberg equilibrium (HWE) tests were calculated with the Arlequin version 2.0 package (University of Geneva, Geneva, Switzerland). χ^2 analysis, Fisher's exact tests, odds ratios (ORs) and statistical power and confidence values were obtained with EpiInfo software (StatCalc) version 6 (Centers for Disease Control and Prevention, Atlanta, Ga., USA). The level of significance for all tests was $p < 0.05$.

Results

As an alternative to using sequencing [19] or hairpin primers [26] to analyze the SNP *DEFB1* 668 C→G, we attempted to design a reliable RT-PCR probe, using Primer Express 2.0 software (Applied Biosystems). However, the high GC content adjacent to the SNP of interest and the size limitations of the probe led us to consider PCR-RFLPs as an alternative, although an assay for this site has been reported using longer probes [15].

Table 2. Comparison of genotype and allele frequencies of patients with AD only and all AD patients

SNP	AD patients with no other allergy (n = 37)							All AD patients (n = 59)						
	geno- type	fre- quency	OR	p value	allelic fre- quencies	OR	p value	geno- type	fre- quency	OR	p value	allelic fre- quencies	OR	p value
<i>DEFB1</i> 668 C→G	CC	3 (8.1)			C = 0.35	2.23 [1.22–4.01]	0.0080	CC	4 (6.8)			C = 0.314	1.88 [1.13–3.13]	0.0285
	GC	20 (54.1)			G = 0.65	0.45 [0.25–0.82]		GC	29 (49.2)			G = 0.686	0.53 [0.32–0.89]	
	GG	14 (37.8)	0.36 [0.16–0.8]	0.0168				GG	26 (44.1)	0.46 [0.24–0.89]	0.0387			
<i>DEFB1</i> 692 A→G	AA	0 (0)			A = 0.28	0.42 [0.23–0.76]	0.0038	AA	0 (0)	0.09 [0–0.09]	0.0087	A = 0.288	0.43 [0.26–0.7]	0.0005
	AG	21 (56.8)			G = 0.72	2.36 [1.32–4.33]		AG	34 (57.6)			G = 0.712	2.31 [1.43–3.78]	
	GG	16 (43.2)	3.21 [1.37–7.34]	0.0063				GG	25 (42.4)	3.09 [1.52–6.26]	0.0015			
<i>DEFB1</i> 1654 G→A	GG	0 (0)			G = 0.46			GG	0 (0)			G = 0.466		
	AG	34 (91.9)	0.06 [0–0.62]	0.0189	A = 0.54			AG	55 (93.2)	0.07 [0–0.69]	0.021	A = 0.534		
	AA	3 (8.1)	17.37 [1.62–860.83]	0.0189				AA	4 (6.8)	13.57 [1.45–645.95]	0.021			
<i>DEFB1</i> 1836 A→G	AA	34 (91.9)			A = 0.96			AA	53 (89.8)			A = 0.949		
	AG	3 (8.1)			G = 0.04			AG	6 (10.2)			G = 0.051		
	GG	0 (0)						GG	0 (0)					

Figures in parentheses are percentages. Figures in brackets indicate 95% CIs. All p values have the Bonferroni correction.

Fourteen of all our patients with AD had an additional form of allergy: 4 had asthma (6.8%), 2 pharmacological allergy (3.4%), 1 allergic conjunctivitis (1.7%), 3 reported allergic rhinitis (5%), and 4 food allergy (6.8%), with no overlap among them. The allele and genotype frequencies of controls and AD patients with overlapping allergies showed no significant differences (table 1). The allele and genotype frequencies of patients with AD only grouped with those with overlapping allergies were compared with controls (table 2). The HWE was altered in both groups of controls and all AD patients for the sites 692 and 1654. We generated haplotypes based on the genotype frequencies of controls and all AD patients. The most likely haplotypes were arbitrarily numbered from 1 to 13 (ht1–13). When considering the 10 most likely predicted haplotypes for both groups, we found that 3 of them, including ht1 (CAAA), ht3 (CAGA) and ht6 (GAAA), were not present in AD patients. Moreover, among the remaining haplotypes, it appeared that the haplotypes ht11 (CGAG), ht12 (CGGG) and ht13 (GGGG)

were not present in controls. This analysis also predicted significant differences in the frequencies of ht5 (CGGA) and ht6 in both groups (table 3).

The estimated linkage disequilibrium suggests that the loci most probably linked in both groups, notwithstanding the approximate 7-kb intronic distance between them, are the SNPs at 692 and 1654 (block 692–1654). Notably, blocks 668–1654 and 692–1836 are probably formed in AD patients (table 4).

To study certain environmental factors, we evaluated some variables that might act as triggers of AD analyzing the correlation between them and the different genotypes. We found a correlation between the duration of breastfeeding and the severity of AD (Spearman's $\rho = 0.346$, $p = 0.009$) as well as the age of AD onset ($\rho = 0.301$, $p = 0.018$). The individual genotypes at each site and when considered all together did not correlate with disease severity according to the eczema area and severity index; neither did they correlate with infection susceptibility (assessed as the number of infections/year) or the

Table 3. Expected haplotypes for controls and all AD patients obtained with the expectation maximization algorithm (Arlequin 2.0) until reaching standard deviation

Haplotype	SNP				Expected frequency		p value	Cases vs. controls	
	668 C→G	692 A→G	1654 G→A	1836 A→G	controls (n = 151)	patients (n = 59)		OR	95% CI
ht1	C	A	A	A	0.009	0	0.636		
ht2	C	A	G	A	0.123	0.138	0.849		
ht3	C	A	G	G	0.003	0	0.489		
ht4	C	G	A	A	0.019	0.054	0.068		
ht5	C	G	G	A	0.041	0.115	0.039	3.25	0.88–12.22
ht6	G	A	A	A	0.086	0	0.040	0.17	0–1.14
ht7	G	A	G	A	0.238	0.133	0.099		
ht8	G	A	G	G	0.014	0.017	0.630		
ht9	G	G	A	A	0.385	0.471	0.230		
ht10	G	G	G	A	0.081	0.038	0.191		
ht11	C	G	A	G	0	0.009	0.280		
ht12	C	G	G	G	0	0.005	0.489		
ht13	G	G	G	G	0	0.019	0.280		

Standard deviation = 0 (50,000 iterations). Fisher's exact test was computed when expected values in a cell were less than 5.

Table 4. Estimation of linkage disequilibrium in all patients with AD based upon haplotype inference (100,000 permutations)

A	668	692	1654	B	668	692	1654
692	0.925			692	0.105		
1654	0.125	0.00002		1654	0.047	0.006	
1836	0.623	0.530	0.775	1836	0.157	0.001	0.354

p values of exact tests show the probability of independent segregation in controls (A) and patients (B).

number of relapsing episodes/year. None of the remaining analyzed variables displayed any significant correlations, such as family history of other allergies, tetanus toxin vaccination and passive or active maternal smoking during pregnancy. Even though *DEFB1* 1836 A→G was not informative for the presence of AD (table 2), allele A correlated with an earlier age of AD onset ($\rho = 0.271$; $p = 0.03$), suggesting an additive effect with the duration of breastfeeding (data not shown). However, we were unable to predict the age of AD onset with both the *DEFB1* 1836 A→G genotype and the duration of breastfeeding, because none of the regression models proved to be significant ($p > 0.1$, data not shown). This phenomenon may be explained by the lack of information with regard to other loci and additional environmental factors not studied.

Discussion

We propose that the *DEFB1* gene is an additional candidate that may be implicated in inflammatory processes involved in the establishment and persistence of AD. Diminished expression of other antimicrobial peptides such as LL-37, hBD-2 [27, 28], hBD-3 [29] and dermcidin [30] in patients with AD might explain an increase in their susceptibility to infection and subsequently to disease worsening. However, this does not explain AD susceptibility itself. Furthermore, variants in *IL10* [31] and *CCL11* (eotaxin) [32] could be responsible for the elevated immunoglobulin E levels in extrinsic AD, but not for the risk of suffering from both extrinsic and intrinsic AD. Thus, we searched for other informative markers by ana-

lyzing four SNPs with a potentially functional repercussion in *DEFB1*, whose peptide product hBD-1 hypothetically acts upstream in the cascade of events leading to AD, because hBD-1 is chemotactic for DCs [5]. In addition, hBD-1 could act as a maturing and activating agent for these cells (through the CCR6 chemokine receptor and Toll-like receptors, respectively) [6, 33], being important regulators of allergic immune responses [1, 34].

With respect to the alterations observed in the HWE, these are often not reported in association studies despite the fact that deviations are very common [35]. These alterations could reveal that one or more of the assumptions in the HWE do indeed influence the genotypic frequencies including positive/negative selection in disease-causing genes [36]. If the sites 692 and 1654 are under selection forces independent of AD status, explained by the important role of hBD-1 in immune protection, or if the deviation is explained by the linkage itself, remains to be demonstrated. As another possible explanation for defensins at the 8p23.1 locus, deviation from HWE has been observed in a population without disease due to duplicons [37]; nevertheless, *DEFB1* has been defined as a unique copy gene [38].

The four SNPs studied have been candidates for studies of infection susceptibility [15, 39], and two of them (668 and 692) for allergic diseases [14]. Differential *DEFB1* expression between individuals [40] could be explained by the presence of one or more SNPs that induces overexpression, as might be the *DEFB1* C→G 668 (also known as *DEFB1* -44 C→G according to the initiation codon-based nomenclature) that generates a putative binding site for nuclear factor- κ B [13]. The proposed effect of this SNP could partially explain the differences in susceptibility to *Candida* sp. colonization in oral mucosa [15] and to perinatal HIV infection [39]. Polymorphisms in terms of gene copy number (duplicons) as for other β -defensins are unlikely to be the explanation [38]. Notwithstanding, we found no correlation between the polymorphisms studied and infection susceptibility in patients with AD. We found that the GG genotype appears to protect against AD (OR = 0.6) and, surprisingly, that G is the major allele in both groups. Nevertheless, the *DEFB1* 668 C→G allele is significantly underrepresented in patients with AD (OR = 0.45). If this SNP does indeed upregulate hBD-1 expression, this result could account for the increased susceptibility of these patients to infections. The C allele could diminish the maturation potential for DCs, explained by a low number of hBD-1 molecules produced in comparison with the G allele. Indeed, the maturation status of DCs has been proposed as an important factor

in the allergic/autoimmune 'decision' of the immune system [41].

The analysis of the *DEFB1* 692 A→G (or *DEFB1* -20 A→G) genotypes suggest that GG is associated with AD susceptibility (OR = 3.21) while AA was not found in any patient. This SNP was studied due to the fact that it is located adjacent to the signal peptide sequence, but its functional repercussion is not known.

In *DEFB1* 1654 G→A genotypes, AA was associated with susceptibility to AD (OR = 17.37). The substitution V37I in hBD-1 could alter the stability of the peptide because this site is adjacent to a Cys residue where one of the three hBD-1 disulfide bridges is anchored. The altered structure could affect the chemotactic properties of hBD-1 [42], and this is consistent with the association of this SNP with the altered hallmark inflammation of DA. The absence of GG genotype at the 1654 site in both groups can be explained by (1) a selection favoring the heterozygotes, (2) a substructured population, and (3) a genotype misassignment. We reject the latter, based on the use and validation of two different genotyping methods in our analysis.

The *DEFB1* 1836 A→G SNP was not informative for the development of AD, although it was correlated with age of AD onset. This putative poly-A site could alter the mRNA stability and eventually the elevated/diminished translation rate of *DEFB1*. In turn, this might favor the surpassing of the homeostatic threshold leading to the earlier onset of the disease. Additionally, the estimated linkage disequilibrium suggests that the rate of recombination between the 692 and 1836 sites was less frequent in AD patients than in controls (linkage of $p = 0.53$ in controls vs. $p = 0.001$ in patients).

Studying the inferred haplotypes revealed that two are overrepresented, either in controls (protective combination ht6, GAAA; $p = 0.04$, with OR = 0.17) or in patients (susceptible combination ht5, CGGA; $p = 0.039$, with a 3.25-fold risk of developing AD). Excluding the noninformative 1836 site, each group has the opposite variant at the sites at which genotype frequencies have highly significant statistical differences (668, 692 and 1654).

Environmental factors have also been considered as important allergy/atopic triggers [1, 43]. Indeed, we found a positive correlation between prolonged breastfeeding and AD severity as well as earlier age of AD onset. Although it is well known that patients with AD, mainly infants, may develop allergies to foods like milk [44], the correlation between the duration of breastfeeding in AD and AD severity that we found is suggested only in few previous reports; additionally, controversy

remains about the effect of maternal lactation in AD [45].

In this study, we have uncovered the association between three SNPs of *DEFB1* and AD and one SNP and the age of AD onset. Unfortunately, the small number of patients with overlapping allergies limits us to perform a correlation and association analysis between subgroups. Nevertheless, excluding these patients, we were able to confirm that the genotypes and alleles are associated with DA and not with asthma or other allergies.

The biological relevance of our findings remains to be elucidated. It will be necessary to study the functional effects of these SNPs on *DEFB1* in inflammatory and allergic diseases as well as to discriminate between genes that cause or modify AD, despite this being a complex trait (we currently think that examples are the *DEFB1* and *IL10* genes, respectively). In addition, we stress the importance of assessing genetic components in combination with the environmental factors that trigger AD. This will be fun-

damental in order to be able to predict susceptibility to AD in the near future, and this information will also help to propose adequate prophylaxis as well as to design useful therapeutic options that will improve the quality of life of patients with AD.

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