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DEFB4A Promoter Polymorphism Is Associated with Chronic Periodontitis: A Case–Control Study

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Background: Human β-defensin-2 is an antimicrobial peptide with antibiotic properties secreted by the oral cavity to protect the host against microbial attack. The inter-individual differences in defensin expression profiles due to genetic variation might be partly responsible for differences in disease susceptibility.
Aims: The objective of this study was to examine whether variation in the human β-defensin-2 gene (*DEFB4A*) is associated with chronic periodontitis (CP).
Materials and Methods: This case-control study used Sanger sequencing to analyze two promoter polymorphisms of the *DEFB4A* gene with potential functional consequences using DNA samples collected from 200 unrelated individuals.
Results: The *DEFB4A rs1339258595* promoter polymorphism is associated with CP risk and clinical attachment level (CAL) but the *rs3762040* polymorphism is not. Carriers of the T allele (*rs1339258595*) were approximately three times less likely to develop periodontitis compared with noncarriers ($p = 0.0004$, odds ratio = 0.35). Consistent with a protective role, the carriers of T allele had a lower CAL compared with the wild-type (G) allele. Moreover, the wild-type diplotype (GGGG) had a significantly higher risk of tooth loss compared with other diplotypes ($p = 0.016$).
Conclusion: This study demonstrates that genetic variation in the promoter region of *DEFB4A* likely affects resistance to periodontal infection and might be a potential marker for CP risk and severity.

Keywords: periodontal disease, genetic susceptibility, multisite variant, promoter, association

Introduction

Chronic periodontitis (CP) is a multifactorial disease influenced by genetic and environmental factors, together with the presence of microbial deposits (Kinane and Mark Bartold, 2007). CP is the result of a change in the balance between microbial dental plaque and the host's response to these microorganisms (Kinane, 2001). Although microbial factors are responsible for CP, the destruction of periodontal tissues is due to the host mounting a defense against these factors (Kinane, 2001). The various cytokines, enzymes, and inflammatory mediators involved in this defense system are under the host's genetic control (Kinane, 2001).

Several studies on twins have suggested that ~50% of the risk for CP may be attributable to genetic factors (Michalowicz *et al.*, 2000). The genetic basis of CP is complex, possibly involving multiple genes as well as interactions between genes and environmental factors (Laine *et al.*, 2012; Loos *et al.*, 2015). Despite the evidence for the substantial genetic effect in the etiology of CP, to date, there is no established specific candidate gene of susceptibility for CP. Identifying genes for CP could help researchers to prevent and develop therapies for periodontitis; it might also aid in recognition of patients requiring more comprehensive therapy.

Antimicrobial peptides are part of the innate immune system and are gene-encoded natural antibiotics (Dale and Fredericks, 2005; Bevins, 2006). Defensins are the most prominent members of a large family of antimicrobial peptides, with studies identifying six types of human α-defensin and four types of human β-defensins (hBDs). hBDs have been mapped to a locus on chromosome 8p22-23.1 (Harder *et al.*, 1997). While α-defensins are primarily expressed in neutrophils and Paneth cells, β-defensins are produced in epithelial tissues located where pathogens make first contact and entry, including the gingiva, gingival crevicular fluids and saliva (Mathews *et al.*, 1999; Dale and Fredericks, 2005; Kohlgraf *et al.*, 2010).

Several lines of evidence support the role of hBD-2 in periodontal diseases. hBD-2 displays microbicidal activity

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against predominantly Gram-negative bacteria, which are the main etiologic agents in periodontal disease (De Smet and Contreras, 2005; Gursoy and Könönen, 2012). Previous studies demonstrated that hBD-2 was involved in gingival inflammation and periodontal disease (Dommisch *et al.*, 2005; Ebrahem, 2013; Ertugrul *et al.*, 2014). Indeed, one *in vitro* study reported the interindividual differences of defensin gene (*DEFB4A*), expression patterns in epithelial cells in response to challenge by oral biofilms (Eberhard *et al.*, 2008). Furthermore, any change in the functioning or quality of defensins increases human's susceptibility to diseases; these changes in the expression of defensins have been linked to genetic factors in the host (Hollox *et al.*, 2003; Groth *et al.*, 2010; Schaefer *et al.*, 2010; Jaradat *et al.*, 2013).

Given the oral cavity's constant exposure to commensal and pathogenic microorganisms, the variation in *DEFB4A* may cause changes in the periodontal sulcus' antibacterial effectiveness. In an attempt to identify useful markers that could predict susceptibility to periodontal disease, this study investigated promoter polymorphisms of *DEFB4A* with potential functional consequences.

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Materials and Methods

Study population

One hundred random cases of CP and 100 control cases were recruited from the patient pool at Ondokuz Mayıs University, School of Dental Medicine in the Black Sea region of Turkey. Participants were informed of the nature and procedures of the study, and informed consent was obtained from each patient in accordance with the Helsinki Declaration. The Research Ethics Committee of Ondokuz Mayis University approved this study (2014/556-630[2013/416-447]). Demographic details, medical and dental history, and smoking status for each subject were recorded (Table 1). A subject was considered to be a nonsmoker if he or she had never smoked or had stopped smoking more than 5 years previous to the date of the study's examination. None of the subjects had a history or current manifestation of systemic disease. Participants with systemic diseases, smokers, pregnant or lactating women, use of antibiotics or anti-inflammatory drugs within 6 months, periodontal treatment within 12 months, or subjects younger than 18 years were excluded from the study.

Table 1. Clinical and Demographic Data

Clinical parameters	*CP,* m ± *SD (Cheng* et al., *2003)*	*Controls,* m ± *SD (Cheng* et al., *2003)*	p
PI	1.48 ± 0.36	0.82 ± 0.46	<0.001
GI	1.01 ± 0.20	0.63 ± 0.45	<0.001
PPD	2.58 ± 0.52	1.58 ± 0.48	<0.001
CAL	2.82 ± 0.55	1.61 ± 0.46	<0.001
OHI	2.53 ± 0.65	1.24 ± 0.69	<0.001
BOP	0.60 ± 0.23	0.41 ± 0.25	<0.001
Age	41.92 ± 10.56	32.35 ± 10.46	<0.001
Gender, male, %	45	36	0.25
Smoking, yes, %	19	12	0.24

BOP, bleeding on probing; CAL, clinical attachment level; CP, chronic periodontitis; GI, gingival index; *m*, mean; OHI, oral hygiene index; PPD, periodontal probing depth; PI, plaque index; SD, standard deviation.

All of the participants were evaluated clinically and radiographically. Specifically, patients were given a thorough periodontal examination, including probing depth (PD), clinical attachment level (CAL) (Michalowicz *et al.*, 2000), bleeding on probing (BOP) (presence or absence), plaque index (PI) (Silness and Löe, 1964), gingival index (GI) (Löe and Silness, 1963), and modified oral hygiene index (Greene and Vermillion, 1964). Measurements were taken and recorded with a Williams probe at six sites (Hu-Friedy). The number of remaining teeth was counted. Subjects without a history of periodontitis and no sites with a PD and CAL >3 mm concomitantly were included in the control group; no restrictions was applied regarding levels of plaque or gingivitis (Garlet *et al.*, 2012). Diagnosis and classification of CP were performed according to the criteria established by the American Academy of Periodontology (Armitage, 1999). Subjects exhibiting radiographic evidence of alveolar bone loss and at least four teeth with a PD ≥4 mm and BOP concomitantly were entered into the CP group.

DNA extraction and genotyping

Two milliliters of peripheral venous blood samples were obtained from each study subject, collected in EDTA-treated vacutainers; they were transferred into falcon tubes containing EDTA and stored at −80°C. Genomic DNA was extracted according to the manufacturer's instructions using the DNA Extraction Kit (Genoks, Ankara, Turkey).

The DNA concentration was determined by ultraviolet spectrophotometry. The genotypes for the *rs3762040* polymorphism and surrounding region in the promoter area of *DEFB4A* were screened for this study. This polymorphism was chosen because the previous work (Kusano *et al.*, 2005) suggested that these variants could be functionally relevant due to its location at the consensus sequence for NF-IL6 binding. A 300 bp fragment of the *DEFB4A* gene was generated using a forward primer (5′-CCCATGTCTCTGTCAC TGCT-3′) and a reverse primer (5′-GGTCTCCCTGTTGGT GATGG-3′) in a thermocycler (Bio-Rad T100 Thermal Cycler, CA). Polymerase chain reaction (PCR) was carried out in a total volume of 50 mL, containing 50 ng of genomic DNA, 10 μM of each primer (Macrogen, Inc., South Korea), 200 mM of dNTP (DN001-0250; GeneDireX), 5 μL of 10 × reaction buffer (2 mM $MgCl_2$), and 1.25 U of Taq polymerase (MB101-0500; GeneDireX). The reaction conditions and cycling parameters were denatured for 4 min at 94°C, followed by 35 cycles of 40 s of denaturing at 94°C, a 1 min annealing at 60°C, a 2 min extension at 72°C, and one cycle at 72°C for 5 min. The PCR amplicons were checked by 1% agarose gel electrophoresis. The PCR products were purified using the GeneDireX PCR Clean-Up and Gel Extraction Kit (NA006-0100; GeneDireX) according to the manufacturer's instructions. The resulting samples were stored at −20°C until sequencing. Screening for polymorphisms was performed using the Sanger sequencing method. DNA templates were processed for the DNA sequencing reaction using the Big Dye Terminator v3.100, the Big Dye Terminator Cycle Sequencing Kit (4337455; Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. This kit includes terminating nucleotides.

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Finally, nucleotide sequences were analyzed with the FATSA search and Chrome-Lete software. Nucleotide sequences were visualized using Chromas (Ver. 1.45) (Fig. 1).

Statistical analysis

Allele frequencies were calculated with the allele counting method. Goodness of fit to Hardy–Weinberg expected proportions was examined through chi-square test. The differences in genotype frequencies between cases and controls were tested using chi-square tests. When any expected number in the 2×2 contingency table was <5, the *p*-value was directly calculated using Fisher's exact test. The pair-wise linkage disequilibrium (LD) between markers was estimated using the D′ method (Lewontin, 1964). Data were expressed as the mean ± standard deviation (SD) (Cheng *et al.*, 2003) or as frequencies of the parameter being evaluated. Multiple logistic regression models were used to examine the association of each genetic marker with disease status. The estimated odds ratios (ORs) were adjusted for the effects of significant covariates such as age, sex, and smoking status. All the computations were performed with R software (Ihaka and Gentleman, 1996). Significance was set at 5% ($p < 0.05$).

Results

A total of 200 unrelated subjects consisting of 100 CP and 100 controls were recruited from the School of Dentistry, Ondokuz Mayıs University. Informative genotyping results were available in 97 CP cases and 88 controls. The genomic DNA obtained from 15 participants was insufficient in quality and quantity to determine the defensin variation; therefore, the final sample comprised 185 subjects. By sequencing a 300 base-pair fragment of the *DEFB4A* gene surrounding *rs3762040* (at position −1029), three polymorphisms were detected in this region: *rs1339258595* (−1037 promoter region), *rs3762040*, and *rs545477125*. *rs545477125* was only identified in 4 subjects out of 185 samples—1 in the CP group and 3 in the control group. Due to low frequency (1.1%), *rs545477125* was excluded from analysis. Pair-wise LD analysis between DEF4A polymorphism revealed significant association ($p < 0.0001$) and D′ values were 0.99 between *rs1339258595* and *rs3762040*.

In this study, genotype frequencies showed that *rs3762040* and *rs1339258595* do not meet the assumption of the Hardy–Weinberg equilibrium (HWE) both in control and cases (HWE; $p < 0.05$). Deviations from HWE usually show genotyping errors, population stratification, or some other artifact. However, the existence of a copy number variation (CNV) can also cause genotype deviation from the HWE (Lee *et al.*, 2008; Plengvidhya *et al.*, 2015). *DEFB4* gene exhibits CNV, which could explain why our cohort of subjects does not meet with the HWE. Excessive heterozygotes seen in the studied genotypes are also an indication of CNV.

The participants' demographic and clinical characteristics are presented in Table 1. The mean age of the participants was 41.92 ± SD 10.56 years, ranging from 20 to 62 years for the CP cases; the mean age was 32.35 ± SD 10.45 years, ranging from 20 to 63 years for the controls. On average, the CP cases were older than the controls ($p < 0.001$). No statistically significant differences were detected in the two groups regarding smoking status. The distribution of individuals for gender and smoking status did not differ between the two groups (Table 1).

Distribution of polymorphisms

The overall genotype distribution of the *DEFB4A* *rs3762040* polymorphism was similar between the CP cases

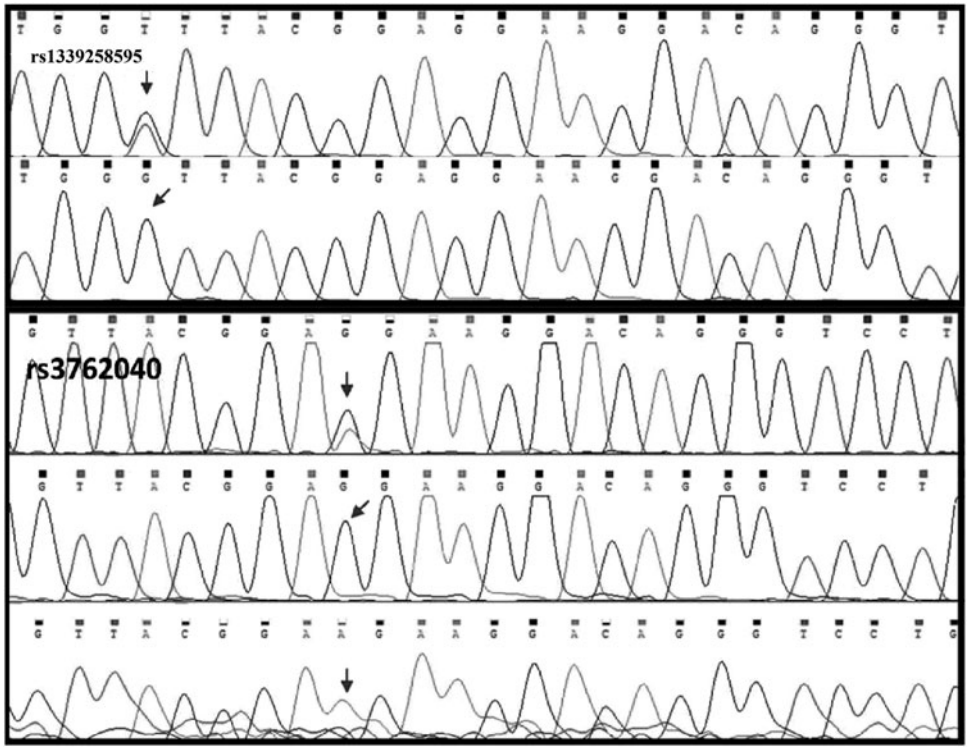


FIG. 1. Sequence variation of *DEFB4A* *rs1339258595* and *rs3762040*. The *arrows* indicate the positions of sequence variation.

Table 2. Genotype and Allele Frequencies of the *rs3762040* Promoter Polymorphism of the *DEFB4* in Chronic Periodontitis Cases and Controls

All Genotypes	*Genotype*				p	
	GG, n *(%)*	*GA,* n *(%)*	*AA,* n *(%)*	*G/A allele (%)*	*Allele*	*Genotype*
Control	37 (43.53)	48 (56.47)	0 (0)	72/28	1	0.72
Cases	42 (44.68)	51 (54.26)	1 (1.06)	72/28		
	Age, sex, smoking status: Adjusted OR GG vs. GA+AA 0.92 (0.48–1.78; $p=0.81$) Unadjusted OR GG vs. GA+AA 0.9 (0.50–1.62; $p=0.72$)					
Nonsmoker						
Control	34 (44.74)	42 (55.26)	0 (0)	72/28	0.66	0.93
Cases	30 (41.66)	41 (56.94)	1 (1.39)	70/30		
	Age, sex: Adjusted OR GG vs. GA+AA 1.14 (0.56–2.31; $p=0.725$) Unadjusted OR GG vs. GA+AA GG vs. GA+AA 1.03 (0.54–1.96; $p=0.927$)					

OR, odds ratio.

and the controls ($p=0.72$, Table 2). The allele frequency of the A allele was similar in both groups ($p=1$). The age-, gender-, and smoking status-adjusted OR for the *DEFB4A* GG genotype versus the GA+AA genotypes was 0.92 (0.48–1.78, $p=0.81$; Table 2). Smoking status was taken into consideration during data analysis to avoid population stratification effects. When stratifying the data by smoking status, there was no statistically significant difference in genotype or allele distribution among nonsmokers ($p=0.93$; Table 2).

In contrast, The *DEFB4A rs1339258595* polymorphism showed a significant difference in both allele ($p=0.005$) and genotype ($p=0.002$) frequencies between the CP cases and the controls. The frequency of the wild-type −1037 G allele was significantly higher in the CP cases than in the controls (89.2% vs. 78.4%, $p=0.005$). The age-, sex-, and smoking status-adjusted OR for the GT versus GG genotypes was 0.35 (0.17–0.72, $p=0.004$). Simply put, carrying a copy of the variant allele of the *DEFB4A* marker −1037 (GT) decreased the patient's risk of CP more than twofold. Stratifying the data by smoking status did not change the results. Similar to the overall analysis, the promoter polymorphism data among nonsmokers revealed that there was a significant association between the *rs1339258595* promoter polymorphism and CP. The distribution of the two *DEFB4A* single nucleotide polymorphisms (SNPs) is presented in Table 3.

Diplotype analysis and CP risk

In addition to single-site analysis, two-site diplotype analysis was also performed. Diplotype analysis was used because genotype-based and diplotype-based analyses stay valid in the presence of Hardy–Weinberg disequilibrium (Zuo *et al.*, 2014). The two-site *DEFB4A* diplotype distributions in the CP cases and the controls are presented in Table 4. Five diplotypes were observed (GGGG, GGGT, AAGG, GAGG, and GAGG). There was only one CP case featuring the AAGG diplotype; therefore, this diplotype was included in the GA heterozygous group. The GGGG diplotype was set as the reference group for comparison purposes since it carried wild-type alleles. Consistent with the genotype analysis, the individuals with the GGGT and GAGT diplotypes versus other diplotypes were associated with a decreased susceptibility to CP (OR = 0.22, 95% CI 0.09–0.58, $p=0.002$ and OR = 0.29, 95% CI 0.10–0.83, $p=0.022$, respectively). Additionally, diplotype-based logistic regression analyses were fitted, with age, gender, and smoking as

Table 3. Genotype and Allele Frequencies of the *rs1339258595* Promoter Polymorphism of the *DEFB4*A in Chronic Periodontitis Cases and Controls

All Genotypes	*Genotype*				p	
	GG, n *(%)*	*GT,* n *(%)*	*TT,* n *(%)*	*G/T allele (%)*	*Allele*	*Genotype*
Control	50 (56.82)	38 (43.18)	0 (0)	78.4/21.6	0.005	0.002
Cases	76 (78.35)	21 (21.65)	0 (0)	89.2/10.8		
	Age, sex, smoking status: Adjusted OR GG vs. GT+TT 0.35 (0.17–0.72; $p=0.004$) Unadjusted OR GG vs. GT+TT 0.36 (0.19–0.69; $p=0.002$)					
Nonsmoker						
Control	42 (55.26)	34 (44.74)	0 (0)	89/11	0.01	0.005
Cases	60 (76.92)	18 (23.08)	0 (0)	78/22		
	Age, sex: Adjusted OR GG vs. GT+TT 0.34 (0.16–0.73; $p=0.006$) Unadjusted OR GG vs. GT+TT 0.37 (0.19–0.74; $p=0.005$)					

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Table 4. Distribution of Promoter Diplotypes Based on Periodontal Disease Status

	GAGG	*GAGT*	*GGGG*	*GGGT*
Control	37 (42%)	14 (15.9%)	13 (14.8%)	24 (27.3%)
CP	43 (46.2%)	9 (9.7%)	29 (31.2%)	12 (12.9%)
				$p=0.009$

confounding factors. Age-, gender-, and smoking status-adjusted diplotype-based logistic models suggested that individuals with GGGT and GAGT diplotypes had a decreased risk of PD (OR = 0.27, 95% CI 0.09–0.78, $p=0.016$ and OR = 0.27, 95% CI 0.08–0.88, $p=0.029$, respectively).

Association between and CP-related quantitative traits

The association of *DEFB4A* with quantitative traits related to CP was also examined—namely, PD, CAL, number of teeth present in the mouth, GI, and PI (Table 5). Interestingly, the analysis provided evidence for an association between *DEFB4A* genetic variants (Table 5). Individuals with the *rs1339258595* GT genotype had a lower mean CAL compared with those with the GG wild-type genotype (1.98 mm vs. 2.36 mm, $p=0.002$). Similar to the single-site analysis, the diplotype analysis showed that individuals with GAGT or GGGT diplotypes had lower CAL compared with the other diplotype carriers ($p=0.006$, Table 6). Adjusting for age, gender, and smoking status did not alter this association ($p=0.001$).

An association trend was observed between the number of teeth present in the mouth and the *rs1339258595* variant ($p=0.09$). Consistent with the protective role of T allele carriers, individuals with the GT genotype had more teeth present in their mouths (Table 5). In line with this result, the diplotype analysis revealed that the wild-type GGGG diplotype carriers had the lowest number of teeth present in their mouths compared with the other diplotype carriers ($p=0.03$, Table 6). Adjustment for age, gender, and smoking status did not alter this association ($p=0.001$).

Discussion

hBD-2 is an antimicrobial peptide expressed by epithelial cells in response to bacterial challenge or inflammation. In the oral cavity, they form important components of the innate immunity against continuous bacterial challenge. Through their chemotactic activity, they form the link between the innate and the acquired immune system. Therefore, polymorphisms in these genes are potential modifiers of the risk and severity of chronic inflammatory periodontal disease. Indeed, researchers have reported the interindividual differences of epithelial cells' defensin expression profiles in response to challenge with oral biofilms (Chadebech *et al.*, 2003; Joly *et al.*, 2005; Vardar-Sengul *et al.*, 2007; Eberhard *et al.*, 2008) that may reflect differences in individuals' susceptibility to developing chronic inflammatory periodontal diseases. Moreover, according to cell line studies, genetic variation accounts for 50% of the observed variation in hBD-2 gene (*DEFB4A*) expression (Hollox *et al.*, 2003; Groth *et al.*, 2010).

Table 5. Mean Quantitative Traits (±Standard Deviation) Among Genotypes

Genotypes	*Homozygote, mean ± SD*	*Heterozygote, mean ± SD*	p[a]
rs1339258595			
CAL	2.36 ± 0.84	1.98 ± 0.60	0.002 (0.001)
PD	2.16 ± 0.72	1.98 ± 0.65	0.12 (0.09)
Teeth present	24.3 ± 4.4	25.4 ± 3.4	0.09 (0.07)
rs3762040			
CAL	2.28 ± 0.82	2.19 ± 0.77	0.76 (0.66)
PD	2.14 ± 0.69	2.06 ± 0.73	0.73 (0.70)
Teeth present	24.14 ± 4.5	25.2 ± 3.65	0.19 (0.14)

[a]Age, gender, smoking adjusted *p*-values.
PD, probing depth.

Table 6. Mean Quantitative Traits (±Standard Deviation) Among Diplotypes

Diplotypes	*CAL, mean ± SD*	*PD, mean ± SD*	*Teeth present, mean ± SD*
GGGG[a]	2.54 ± 0.88[b,d]	2.24 ± 0.70	23.14 ± 4.97
GGGT[b]	1.97 ± 0.63[a]	2.00 ± 0.66	25.31 ± 3.66[a]
GAGG[c]	2.25 ± 0.81	2.09 ± 0.75	25.12 ± 3.81[a]
GAGT[d]	1.99 ± 0.57[a]	1.96 ± 0.66	25.55 ± 2.99[a]
p	0.006	0.508	0.031
*p**	0.001	0.273	0.016

*Age, gender, smoking-adjusted *p*-values.
Different letters indicate significant differences ($p<0.05$).

Previously, Kusano *et al.* (2005) showed that genetic variation within promoter region at position *rs3762040* (−1029) of the *DEFB4A* may alter the production of the hBD-2 protein by using luciferase reporter assay. This SNP is located at a potential binding site for the transcription factor NF-IL6 (the nuclear factor interleukin-6). The authors reported that the wild-type (G) allele of *rs3762040* conferred decreased transcriptional activity than did the variant type (A). Owing to its possible role in the *DEFB4A* protein (hBD-2), the present study investigated possible associations between *rs3762040* polymorphisms and periodontitis susceptibility, screened for neighboring variants. Contrary to expectations, in the present study, *rs3762040* polymorphism did find any significant association with CP risk neither in alleles nor in genotypes. In contrast, a neighboring polymorphism, *rs1339258595*, showed a significant association with CP. Individuals presenting with the T allele were 2.86 times less likely to develop periodontitis. This association was independent of the periodontitis-specific covariates of age, gender, and smoking (with an OR of 0.35, $p=0.004$). Additionally, two-site diplotype analysis also confirmed that individuals with the GGGT and GAGT diplotypes had a decreased risk of periodontal disease and adjusting for an age, gender, and smoking status did not alter this association.

To the present researchers' knowledge, there has only been one previous study on the association of *DEFB4A* with periodontal disease. Jaradat *et al.* (2013) investigated the relationship between a CNV in *DEFB4A* and CP in a French–German population. Their results pointed to an association between decreased copy number and disease severity. These results support the hypothesis of the present study that β-defensin-2 may be a candidate gene in CP. However, it was not possible to directly compare the findings of Jaradat *et al.* (2013) with those of the present study, as they did not

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examine SNPs of the *DEFB4A* in their study. Interestingly, although different polymorphisms of *DEF4A* were studied, the effect size of CNV in Jaradah's study (OR = 3) was compared with the present study's reported effect size for the *rs1339258595* variant (OR = 2.8).

In addition to CNV, sequence variations are possible phenotype determinants. Fredman *et al.* (2004) described a new type of polymorphism multisite variation (MSV) for SNPs in duplicons. MSV represents the sum of the signals from many individual duplicon copies that vary in sequence content due to duplication, deletion, or gene conversion; researchers have proposed that they determine phenotypes like SNPs do. Fredman *et al.* (2004) and Groth *et al.* (2010) demonstrated that both copy number and sequence variations affect *DEFB4A* expression.

This study tested two cosegregating MSVs for their association with periodontal disease. It found a significant relationship between *DEFB4A* MSV and periodontal disease. Although different polymorphisms in the *DEFB4A* gene were investigated in this study as well as in Jaradah *et al.*'s (2013) study, the parallel results support the role of *DEFB4A* in periodontal disease. Since both studies were independent studies on different populations, the present study's findings that the nucleotide state of an MSV (*rs1339258595*) may affect susceptibility to periodontal disease might show a genuine association. SNP *rs1339258595* itself might have a potential functional effect on decreased susceptibility to periodontal disease. However, it is also possible that this MSV is indirectly associated with periodontal disease and may be in LD with CNV or another sequence variation in an adjacent region of the genome.

In addition to disease risk, this study examined the association of *DEFB4A* MSVs with quantitative measures of CP, including CAL, PD, and remaining teeth in the mouth. Consistent with its protective role, the *rs1339258595* GT genotype was associated with lower mean attachment loss compared with the GG genotype (1.98 ± 0.60 vs. 2.36 ± 0.84, $p = 0.001$; age, gender, and smoking status-adjusted). An association trend was also observed between the remaining teeth in the mouth and the *rs1339258595* genotype ($p = 0.07$). Similar to the single-site analysis, diplotype analysis revealed that diplotypes GGGT and GAGT had lower mean CAL ($p = 0.006$). The marginal association that was seen between remaining teeth and the *rs1339258595* genotype in the single-site analysis became significant in the diplotype analysis. The carriers of the wild-type diplotype GGGG had lower remaining teeth compared with the other diplotypes (23 vs. 25 teeth, $p = 0.03$; Table 5). This result also corresponds with Jaradah *et al.* (2013), who found an association between *DEFB4A* copy number and CAL.

An earlier study (Kusano *et al.*, 2005) sequenced *DEFB4A* gene and identified 10 SNPs in Japanese population ($n = 50$). Interestingly, the allelic frequencies observed in the present study were very similar to those reported in the study by Kusano *et al.* (2005) for both *rs3762040* (28% for rare allele) and *rs1339258595* variant (25%), despite these seemingly different population.

One of the limitations of this study is that the control group was younger than the CP group. Although it is known that DNA does not change with age in terms of polymorphisms (Hodge *et al.*, 2001), the prevalence of CP increases with age (Kinane *et al.*, 2006). This study tried to address this problem by considering potential confounding factors, such as age and gender, in its logistic regression analysis.

Smoking is another established risk factor in the pathogenesis of periodontal disease that alters the host's response to plaque (Kinane *et al.*, 2006). In the study's samples, there was no statistically significant difference between the CP cases and the controls in terms of smoking status ($p = 0.24$). Nonetheless, to adjust for possible confounding effects, smoking was included in a logistic model. Moreover, the data were stratified based on smoking status—similar to the overall analysis, there was a significant association between the *rs1339258595* promoter polymorphism and CP among nonsmokers (Table 3).

The current study was limited by its cross-sectional design; it is not possible to identify the causal relation between periodontitis and *DEFB4A* polymorphisms. Additionally, the study was designed to detect moderate effect size with an OR >2.5 under the assumption of a 11% (Tonetti *et al.*, 2017) disease prevalence, 10% minor allele frequency, complete LD, 1:1 case per control ratio, and a 5% error rate in an allelic test. Therefore, this study did not have the power to detect variants with smaller effect sizes (e.g., 1.3–1.6), which requires larger sample sizes. It is possible that the *DEFB4A rs3762040* polymorphism had a smaller effect size than what this study was capable of detecting. Indeed, diplotype analysis revealed that individuals with GAGG, along with other diplotypes, had higher remaining teeth counts compared with those with the wild-type GGGG diplotype.

However, one of the strengths of this study was the inclusion of gingivitis patients in the control group. No restriction was applied concerning levels of plaque or gingivitis in the control group. The majority of our control group except for two periodontally healthy controls had gingivitis. Garlet *et al.* (2012) suggested including patients with gingivitis as controls because the inclusion of periodontally healthy subjects does not allow the identification of the ''susceptible'' or ''resistant'' phenotypes of the included patients. The use of chronic gingivitis as reference status improves the power and odds of periodontitis genetic studies when compared with the use of periodontitis versus periodontally healthy subjects (Garlet *et al.*, 2012).

Taking the aforementioned limitations of the present study into account, the results suggested that the *rs3762040* polymorphism of the hBD-2 gene was not associated with CP. However, the *rs1339258595* G/T polymorphism seemed to be significantly associated with CP, suggesting that the variant T allele might be a protective factor for CP. Additionally this variant associated with CP-related traits.

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Author Disclosure Statement

No competing financial interests exist.

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References

Armitage GC (1999) Development of a classification system for periodontal diseases and conditions. Ann Periodontol 4:1–6.

Bevins CL (2006) Paneth Cell Defensins: Key Effector Molecules of Innate Immunity. London, UK: Portland Press Limited.

Chadebech P, Goidin D, Jacquet C, *et al.* (2003) Use of human reconstructed epidermis to analyze the regulation of β-defensin hBD-1, hBD-2, and hBD-3 expression in response to LPS. Cell biology and toxicology 19:313–324.

Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW (2003) De-orphanization of Cytochrome P450 2R1 a microsomal vitamin D 25-hydroxylase. J Biol Chem 278:38084–38093.

Dale BA, Fredericks LP (2005) Antimicrobial peptides in the oral environment: expression and function in health and disease. Curr Issues Mol Biol 7:119.

De Smet K, Contreras R (2005) Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol Lett 27:1337–1347.

Dommisch H, Acil Y, Dunsche A, *et al.* (2005) Differential gene expression of human β-defensins (hBD-1,-2,-3) in inflammatory gingival diseases. Oral Microbiol Immunol 20:186–190.

Eberhard J, Menzel N, Dommisch H, *et al.* (2008) The stage of native biofilm formation determines the gene expression of human β-defensin-2, psoriasin, ribonuclease 7 and inflammatory mediators: a novel approach for stimulation of keratinocytes with in situ formed biofilms. Oral Microbiol Immunol 23:21–28.

Ebrahem MA (2013) Expression of human beta defensins (HBDs) 1, 2 and 3 in gingival crevicular fluid of patients affected by localized aggressive periodontitis. Saudi Dent J 25:75–82.

Ertugrul A, Sahin H, Dikilitas A, *et al.* (2014) Gingival crevicular fluid levels of human beta-defensin-2 and cathelicidin in smoker and non-smoker patients: a cross-sectional study. J Periodontal Res 49:282–289.

Fredman D, White SJ, Potter S, *et al.* (2004) Complex SNP-related sequence variation in segmental genome duplications. Nat Genet 36:861.

Garlet GP, Trombone APF, Menezes R, *et al.* (2012) The use of chronic gingivitis as reference status increases the power and odds of periodontitis genetic studies—a proposal based in the exposure concept and clearer resistance and susceptibility phenotypes definition. J Clin Periodontol 39:323–332.

Greene JG, Vermillion JR (1964) The simplified oral hygiene index. J Am Dent Assoc 68:7–13.

Groth M, Wiegand C, Szafranski K, *et al.* (2010) Both copy number and sequence variations affect expression of human DEFB4. Genes Immun 11:458.

Gursoy UK, Könönen E (2012) Understanding the roles of gingival beta-defensins. J Oral Microbiol 4:15127.

Harder J, Siebert R, Zhang Y, *et al.* (1997) Mapping of the gene encoding human β-defensin-2 (DEFB2) to chromosome region 8p22–p23. 1. Genomics 46:472–475.

Hodge P, Riggio M, Kinane D (2001) Failure to detect an association with IL1 genotypes in European Caucasians with generalised early onset periodontitis. J Clin Periodontol 28:430–436.

Hollox EJ, Armour JA, Barber JC (2003) Extensive normal copy number variation of a β-defensin antimicrobial-gene cluster. Am J Hum Genet 73:591–600.

Ihaka R, Gentleman R (1996) R: a language for data analysis and graphics. J Comput Graph Stat 5:299–314.

Jaradat S, Hoder-Przyrembel C, Cubillos S, *et al.* (2013) Beta-defensin-2 genomic copy number variation and chronic periodontitis. J Dent Res 92:1035–1040.

Joly S, Organ CC, Johnson GK, *et al.* (2005) Correlation between β-defensin expression and induction profiles in gingival keratinocytes. Mol Immunol 42:1073–1084.

Kinane DF (2001) Causation and pathogenesis of periodontal disease. Periodontology 2000 2000 25:8–20.

Kinane DF, Mark Bartold P (2007) Clinical relevance of the host responses of periodontitis. Periodontology 2000 43:278–293.

Kinane DF, Peterson M, Stathopoulou PG (2006) Environmental and other modifying factors of the periodontal diseases. Periodontology 2000 40:107–119.

Kohlgraf KG, Pingel LC, Dietrich DE, Brogden KA (2010) Defensins as anti-inflammatory compounds and mucosal adjuvants. Fut Microbiol 5:99–113.

Kusano K, Abiko Y, Nishimura M, *et al.* (2005) Single-nucleotide polymorphism (SNP) in β-defensin 2 in a Japanese population and an effect of-1029 SNP on promoter activity. Oral Sci Int 2:80–84.

Laine ML, Crielaard W, Loos BG (2012) Genetic susceptibility to periodontitis. Periodontology 2000 58:37–68.

Lee S, Kasif S, Weng Z, Cantor CR (2008) Quantitative analysis of single nucleotide polymorphisms within copy number variation. PLoS One 3:e3906.

Lewontin R (1964) The interaction of selection and linkage. I. General considerations; heterotic models. Genetics 49:49–67.

Löe H, Silness J (1963) Periodontal disease in pregnancy I. Prevalence and severity. Acta Odontol Scand 21:533–551.

Loos BG, Papantonopoulos G, Jepsen S, Laine ML (2015) What is the contribution of genetics to periodontal risk? Dent Clin North Am 59:761–780.

Mathews M, Jia HP, Guthmiller JM, *et al.* (1999) Production of β-defensin antimicrobial peptides by the oral mucosa and salivary glands. Infect Immun 67:2740–2745.

Michalowicz BS, Diehl SR, Gunsolley JC, *et al.* (2000) Evidence of a substantial genetic basis for risk of adult periodontitis. J Periodontol 71:1699–1707.

Plengvidhya N, Chanprasert K, Tangjittipokin W, *et al.* (2015) Detection of CAPN10 copy number variation in Thai patients with type 2 diabetes by denaturing high performance liquid chromatography and real-time quantitative polymerase chain reaction. J Diabetes Investig 6:632–639.

Schaefer A, Richter G, Nothnagel M, *et al.* (2010) A 3′ UTR transition within DEFB1 is associated with chronic and aggressive periodontitis. Genes Immun 11:45.

Silness J, Löe H (1964) Periodontal disease in pregnancy II. Correlation between oral hygiene and periodontal condition. Acta Odontol Scand 22:121–135.

Tonetti MS, Jepsen S, Jin L, Otomo-Corgel J (2017) Impact of the global burden of periodontal diseases on health, nutrition and wellbeing of mankind: a call for global action. J Clin Periodontol 44:456–462.

Vardar-Sengul S, Demirci T, Sen BH, *et al.* (2007) Human beta defensin-1 and -2 expression in the gingiva of patients with specific periodontal diseases. J Periodontal Res 42:429–437.

Zuo L, Wang K, Luo X (2014) Use of diplotypes–matched haplotype pairs from homologous chromosomes–in gene-disease association studies. Shanghai Arch Psychiatry 26:165.

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