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Original Research Article

Association between polymorphisms in the gene encoding beta-defensin 1 and gingivitis, in children

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Abstract

Objective: The aim of this study was to evaluate the possible association between genetic polymorphisms in *DEFB1* (rs1799946 and rs11362) and the development of gingivitis in children from the Amazon region of Brazil. **Material and methods:** The study included 27 healthy children, 10-12 years old, from public schools in Manaus. For the oral examination, the parameters described in the gingival index of Löe and Silness were used to identify the presence or absence of gingivitis. The dependent variable was categorized as "Yes" for children with gingivitis (experimental group; n=10) and "No" for children without gingivitis (control group; n=17). Genomic DNA was extracted from saliva, the selected polymorphisms in the *DEFB1* gene (rs1799946 and rs11362) were genotyped by TaqMan PCR and an endpoint analysis was performed. Genotypic and allelic distribution between groups was performed using the Fisher's exact test with an established alpha of 5%. **Results:** It was found that evaluated SNPs in the *DEFB1* gene (rs1799946 and rs11362) were not associated with gingivitis ($p>0.05$). **Conclusion:** The single nucleotide polymorphisms (SNP) of references rs1799946 and rs11362 in the *DEFB1* gene had no function on gingivitis, promoting neither harmful nor beneficial effects, in children.

Introduction

Gingivitis is the most prevalent form of periodontal disease in individuals of all age groups, including children and adolescents [5]. According to Botero *et al.* [5], the frequency of gingivitis in children and adolescents in Latin American countries range from 31 to 56%, with 31% in Brazil. The authors emphasized the importance of preventing and treating severe gingivitis at an early age.

Although the tissue changes induced by gingivitis are reversible [26], this inflammatory condition has particular clinical relevance since it is considered the precursor of periodontitis, which is a disease characterized by gingival inflammation combined with loss of connective tissue and bone attachment [46]. Studies indicate that genetic, microbiological, environmental and immunological factors may influence the transition between gingivitis and periodontitis [34].

According to Yoshie *et al.* [52], Zhang *et al.* [53] and Laine *et al.* [24], genetic susceptibility factors are relevant in periodontal disease (PD). These genetic factors correspond to the multiple genetic variations in molecules involved in the regulation of the immune-inflammatory response, which play a decisive role in the initiation, progression and control of the disease.

Individual genetic variations could influence gene expression and protein production [37]. Thus, the study of DNA sequence variations, called single nucleotide polymorphisms (SNPs), can elucidate the association between genetic variations and phenotype or disease [42].

Human beta-defensins (hBDs) are small cationic peptides with antimicrobial and immune-regulatory functions which participate primarily in the first line of defense against pathogens [15]. Human beta-defensins (hBDs) have bi-directional regulatory relations to the adaptive immune system, angiogenesis and wound healing [44], in addition to having broad microbicidal activity [15].

Expression and secretion of hBDs are dependent on environmental (bacterial and inflammatory stimulation) and genetic factors [35]. In the oral cavity, hBDs are expressed by the oral epithelium, tongue and salivary glands [16], and are released into saliva [18] and gingival crevicular fluid [14, 51]. hBD-1 is secreted constitutively in periodontal tissues [15, 35] and its expression in gingival epithelia may be directly associated with the maintenance of periodontal homeostasis [29]. The role of genetic polymorphisms in the hBD-1 gene (*DEFB1*) in oral health, particularly in gingivitis and periodontal disease, is still not fully elucidated [17, 38]. In addition, although the association between *DEFB1* polymorphisms and PD has been investigated in different populations with controversial results [30, 45, 47], there are no published studies, to date, evaluating the association of these polymorphisms with gingivitis in children, justifying the relevance of the present study.

The early identification of these genetic factors or risk indicators for the development of gingivitis or periodontitis can help in the identification of predisposed individuals, enabling the institution of personalized therapies and more effective preventive strategies [53]. Additionally, knowledge of the genetic influence of these antimicrobial peptides on the development of periodontal disease may enable to benefit from these biomolecules within the context of adjunctive therapy [35].

Therefore, the objective of the present study was to evaluate the possible association of the *DEFB1* polymorphisms (rs1799946 and rs11362) and the development of gingivitis in children.

Material and methods

The study was approved by The Human Ethics Committee of Amazon State University (Nº 923.569). Informed written consent was obtained from the parents and age appropriate assent documents were used for all children.

Participants

The study included 27 healthy children, 10-12 years old, from public schools in Manaus. Manaus is the capital city of the state of Amazonas, located in the Brazilian North Region.

According to the exclusion criteria of the present study, participants with a syndrome, systemically compromised, users of orthodontic appliances, participants who were chronically using medication or used antibiotics in the last 6 months and individuals biologically related (siblings or first cousins) were considered ineligible.

During the anamnesis, socio-demographic, environmental and cultural data were collected from the participants and their caregivers/guardians. Information regarding oral hygiene habits (tooth brushing and flossing), use/exposure to fluorides (use of fluoride toothpaste and use of fluoride mouthwash) and diet were also collected.

The clinical examination was performed by a single calibrated examiner, specialist in pediatric dentistry, with a clinical mirror and periodontal probe in an environment with good natural lighting. For the oral examination, the parameters described in the gingival index of Löe [27] were used to identify the presence or absence of gingivitis.

The new classification system defines gingivitis in two categories, namely, dental plaque-induced gingivitis and non-dental plaque biofilm-induced gingival disease; and three forms of periodontitis including periodontitis, necrotizing periodontitis, and periodontitis as a manifestation of systemic diseases [2]. This systematic process of periodontal diagnosis and classification is important for the establishment of a correct diagnosis and treatment plan, as well as for the study of the etiology, pathogenesis and treatment of these diseases and clinical conditions. In the present study, only dental plaque-induced gingivitis were included.

The evaluation consisted of gentle probing of the gingival sulcus at the four sites (buccal, lingual/palatal, mesial and distal) of all teeth and bleeding analysis. The data obtained were recorded, obtaining the scores of the analyzed index.

Children who had gingival bleeding in at least 10% of the gingival index assessment sites were diagnosed with gingivitis [2]. Thus, the dependent variable was categorized as "Yes" for children with gingivitis (experimental group) and "No" for children without gingivitis (control group).

Collection and processing of biological material

Saliva samples were collected as a source of genomic DNA, following a previously published protocol [22]. Subjects performed a mouthwash with 5 ml of saline solution for 1 minute. The entire volume of the mouthwash was placed in specific 15 ml centrifuge tubes and kept at -20ºC until being sent to the Molecular Biology Laboratory of the Department of Pediatric Dentistry of the School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil.

For processing and analysis, each tube containing the salivary suspension was centrifuged at 550 g for 10 minutes to sediment the cell pellet. The supernatant was discarded in 2.5% sodium hypochlorite and the pellet resuspended in 1 ml of extraction buffer (TE) (10 mM Tris-HCl, pH 7.8; 5 mM EDTA; 0.5% SDS). Subsequently, the biological material was transferred to a 1.5 ml tube and frozen at -20ºC, until DNA extraction.

The samples were defrosted and incubated with 100 ng/ml of Proteinase K in a water bath at 56ºC overnight and subjected to precipitation processes using 400 μL of 10 M ammonium acetate solution. Next, all tubes were manually shaken for 5 minutes and centrifuged for 15 minutes (12000 rpm). The supernatant was divided into two tubes of 700 μL each. The same volume of ice-cold isopropyl alcohol (700 μL) was added to each sample, followed by vigorous hand shaking. The formation of a "DNA cloud" was observed in each specimen of all aliquots that were, subsequently, centrifuged for 20 minutes at 12000 rpm. The supernatant was discarded carefully so as not to displace the DNA pellet, and 1 ml of ice-cold 70% ethanol was added and centrifuged for 15 minutes at 12000 rpm. Subsequently, the supernatant was discarded and the tube was opened and upturned in paper to dry for, at least, 30 minutes and to evaporate the excess of 70% ethanol. The DNA pellet was resuspended in 50 μL of TE and frozen at -20ºC.

Molecular analysis

Genomic DNA was extracted from the saliva samples for molecular analysis according to a previously reported method [22]. Quantification of the concentration and purity of the DNA was determined by a spectrophotometer (Nanodrop 1000; Thermo Scientific, Wilmington, DE, USA).

Polymorphic variations in the *DEFB1* gene were selected and evaluated using the USCS GenomeBioinformatics. The characteristics of the studied polymorphisms are presented in table I. The polymorphisms were blindly genotyped by polymerase chain reactions (PCR) using the TaqMan method (ABI Prism 7900HT, Applied Biosystems, Foster City, CA, USA), and an end-point analysis was performed. The interpretation was performed using software provided by Applied Biosystems (Foster City, CA, USA) for allelic discrimination.

Statistical analysis

The Hardy-Weinberg test was performed to assess the balance of SNPs. Genotypic and allelic distribution between groups was performed using the Fisher's exact test. Adjusted Binary Logistic Regression was also performed. IBM SPSS Statistics for Windows, Version 23.0 software was used for the analyses (Armonk, NY: IBM Corp.). Values of $p<0.05$ were considered significant.

Results

Twenty-seven children with a mean age of 9.92 (DP=0.82) were included in the sample, being 16 boys (59.2%) and 11 girls (40.8%). A total of 10 children (37.1%) were diagnosed with gingivitis and 17 children (62.9%) composed the control group. The biofilm index was significantly higher in children with gingivitis ($p=0.007$). Other sample characteristics are presented in table II and did not show a statistically significant difference between groups.

All SNPs were in Hardy-Weinberg equilibrium (p>0.05). The amplification rate was 100% for all SNPs.

The genotypic and allelic distributions are presented in table III and the adjusted logistic regression analysis is presented in table IV. It was found that evaluated SNPs in the *DEFB1* gene (rs1799946 and rs11362) were not associated with gingivitis (p>0.05).

Table I – Characteristics of the studied SNPs

Gene	SNPs	Base change	Function/position	MAF#
DEFB1	rs1799946	[C/**T**]	Variant 5'UTR	0.36
	rs11362	[C/**T**]	Variant 5'UTR	0.42

Note: Bold means the less frequent allele. # Minor allele frequency. The information are from: https://www.ncbi.nlm.nih.gov/snp

Table II – Sample characteristics

Variables		Gingivitis		
		No	Yes	P-value
Age	Mean (SDψ)	9.82 (0.80)	10.1 (0.87)	0.505#
Gender	Male (%)	10 (58.8)	6 (60.0)	0.637†
	Female (%)	7 (41.2)	4 (40.0)	
Delayed permanent teeth eruption	No (%)	9 (56.2)	4 (40.0)	0.344†
	Yes (%)	7 (43.8)	6 (60.0)	
Sweet ingestion between meals	No (%)	12 (70.6)	5 (50.0)	0.255†
	Yes(%)	5 (29.4)	5 (50.0)	
Nail bite	No (%)	13 (76.5)	6 (60.0)	0.316†
	yes (%)	4 (23.5)	4 (40.0)	
Who brushes the teeth	Children (%)	16 (94.1)	8 (80.0)	0.124§
	Guardian (%)	1 (5.9)	0 (0.0)	
	Both (%)	0 (0.0)	2 (20.0)	
Frequency of teeth brush	2 (%)	4 (23.5)	2 (20.0)	0.903§
	3 (%)	12 (70.6)	8 (80.0)	
	4 or more (%)	1 (5.9)	0	
Dental floss frequency	No (%)	3 (17.7)	4 (40.0)	0.072§
	Sometimes (%)	10 (58.8)	6 (60.0)	
	Every day (%)	4 (23.5)	0 (0.0)	
Type of tooth paste	Adult (%)	13 (76.5)	7 (70.0)	0.525†
	For children (%)	4 (23.5)	3 (30.0)	
Mouthwash use	No (%)	14 (82.4)	7 (70.0)	0.388†
	Yes (%)	3 (17.6)	3 (30.0)	
Body mass index	Mean (SD)	17.5 (4.14)	20.5 (4.43)	0.077#
Biofilm index	Mean (SD)	3.52 (1.25)	5.48 (1.84)	**0.007#**

Note: ψ standard deviation. # Man-Whitney test. † Fisher exact test. § Chi-square test. Bold form means statistical significance difference (p>0.05)

Table III – Genotype and allele distribution among groups

Genes	SNPs	Models	Genotypes	Gingivitis			
				No (%)	Yes (%)	P-value	OR# (IC† 95%)
DEFB1	rs1799946	Co-dominant	CC	7 (41.2)	6 (60.0)		Reference
			CT	8 (47.0)	3 (30.0)	0.422	0.43 (0.09 – 2.65)
			TT	2 (11.8)	1 (10.0)	0.999	0.58 (0.03 – 6.21)
		Recessive	CC + CT	15 (88.2)	9 (90.0)		Reference
			TT	2 (11.8)	1 (10.0)	0.999	0.83 (0.05 – 8.01)
		Dominant	CC	7 (41.2)	6 (60.0)		Reference
			CT + TT	10 (58.8)	4 (40.0)	0.728	0.66 (0.18 – 2.94)
		Allele	C	22 (64.7)	15 (75.0)		Reference
			T	12 (35.3)	52 (25.0)	0.549	0.61 (0.19 – 2.19)
	rs11362	Co-dominant	CC	4 (23.5)	2 (20.0)		Reference
			CT	9 (53.0)	7 (70.0)	0.999	1.55 (0.23 – 9.83)
			TT	4 (23.5)	1 (10.0)	0.999	0.50 (0.02 – 6.09)
		Recessive	CC + CT	13 (76.5)	9 (90.0)		Reference
			TT	4 (23.5)	1 (10.0)	0.621	0.36 (0.02 – 3.11)
		Dominant	CC	4 (23.5)	2 (20.0)		Reference
			CT + TT	13 (76.5)	8 (80.0)	0.999	1.23 (0.21 – 7.57)
		Allele	C	17 (50.0)	11 (55.0)		Reference
			T	17 (50.0)	9 (45.0)	0.783	0.81 (0.25 – 2.50)

Note: # Odds Ratio. † Confidence interval. Fisher exact test was used for comparisons

Table IV – Binary logistic regression analysis

Genes	SNPs	Genotype of reference	Genotypes	Control vs. Gingivitis		
				Coefficient	OR# (IC† 95%)	p
DEFB1	*rs1799946*	CC	CT	-0.13	0.87 (0.08-9.15)	0.991
			TT	-19.3	#	0.999
	rs11362	CC	CT	-0.06	0.94 (0.11 – 7.74)	0.955
			TT	21.63	#	0.999

Note: # Odds Ratio. † Confidence interval. Regression adjusted by age, gender and biofilm

Discussion

Considering the multifactorial etiology of periodontitis [10], its genetic basis is complex, possibly involving multiple genes as well as interactions between genes and environmental factors [28].

The involvement of genetic control in the etiology of gingivitis can be evidenced by studies involving patients with Down Syndrome [40] and twin patients [31]. Michalowicz *et al.* [31] evidenced a significant genetic component for gingivitis and other periodontal clinical parameters, supporting the role of genetics in disease susceptibility.

Thus, the present study aimed to investigate a possible association between polymorphisms (rs1799946 and rs11362) in the human beta-defensin 1 (hBD-1) gene constitutively expressed in the oral mucosa with the presence of gingivitis. These peptides are components of the innate immune response [20, 49], exhibiting immunomodulatory activities and antimicrobial properties, especially against Gram-negative bacteria and fungi [39].

Studies with samples of gingival epithelium have demonstrated changes in the expression patterns of hDBs during inflammatory gingival and periodontal diseases [8]. Gene expression of hBD1 has been detected less frequently in the tissues with gingivitis, compared to the healthy gingival tissue [12].

Costa *et al.* [9] demonstrated significantly higher levels of hBD-1 in the gingival crevicular

fluid of healthy patients compared to subjects with chronic periodontitis. The significantly higher frequency of mRNA transcription of hBD-1 in gingival crevicular fluid from healthy sites of individuals without periodontitis, in relation to expression in diseased sites of patients affected by localized aggressive periodontitis, was evidenced by Ebrahem [13]. Other authors also demonstrated significantly lower expression of beta-defensins (1, 2 and 3) in inflamed tissues, compared to non-inflamed tissues [3, 12, 19, 25].

These previous findings confirm the biological plausibility of the study of Brancatisano *et al.* [6] which suggested an intrinsic inability of individuals with periodontal disease to produce beta-defensins, evidencing a higher expression in healthy individuals. The authors indicated the potential influence of genetic factors such as polymorphisms or different genomic copy numbers of beta-defensins, in individuals with periodontitis.

Genetic polymorphisms are variations in DNA and are related to biodiversity, genetic variation, adaptation and evolution. A part of genetic polymorphisms can exert allele-specific effects on the regulation of gene expression or encoded protein function, promoting individual differences in various biological traits and susceptibility to diseases [1].

Evidence indicates that genes encoding beta-defensin 1 carry multiple single nucleotide polymorphisms (SNPs) [11], being considered potential modifiers of risk and severity of inflammatory periodontal disease [23].

Polesello *et al.* in 2015 [37] demonstrated a significant association between healthy individuals who had different polymorphisms in the *DEFB1* gene at positions -52 (rs1799946) and -44 (rs1800972) and salivary concentrations of hBD1, that is, the genotypic variation G/G at position -52 showed higher levels of protein than G/A and A/A and individuals with the C/G variation at position -44 also showed a higher protein concentration than the homozygous C/C type. On the other hand, the -20G > A (rs11362) polymorphism had no influence on the salivary levels of hBD1. The authors concluded that variations in the *DEFB1* gene may be effectively involved in the regulation of hBD1 production in saliva. Agreeing with this observation, a recent meta-analysis [7] reported that SNPs in the *DEFB1* gene have functional effects, affecting the expression and function of hBD1.

Regarding the association with gingivitis, there are no studies in the literature investigating the functional role of genetic variants of the *DEFB1* gene in the susceptibility to the development of this inflammatory condition, characterizing the uniqueness of the present study. Our results showed that the single nucleotide polymorphisms (SNP) of references rs1799946 and rs11362 in the *DEFB1* gene had no function on gingivitis, promoting neither harmful nor beneficial effects. Thus, indirect correlations can be made with the specific literature related only to periodontitis.

Recently, an increasing number of studies have focused on the association between polymorphisms in the *DEFB1* gene and periodontitis, showing inconsistency in some results. Two recent meta-analyses [7, 54] demonstrated that the rs11362, rs1799946 and rs1800972 polymorphisms in the *DEFB1* gene were not associated with periodontitis. The absence of association between these polymorphisms and the development of chronic periodontal disease was also evidenced by Schaefer *et al.* [41] and Shao *et al.* [43]. The findings of the present study confirmed this lack of functional effects of polymorphisms in the *DEFB1* gene also for gingivitis, in children.

Similarly, the results of Boniotto *et al.* [4] indicated that the SNP at position -44 (rs1800972) in the *DEFB1* gene had a similar distribution between healthy patients and those with early-onset periodontal disease. As in the present study, these authors showed no association, although the SNP evaluated was different.

On the other hand, Zupin *et al.* [55] revealed that the rs11362 and rs1800972 SNPs in the *DEFB1* gene were significantly related to periodontitis. Similarly, according to the meta-analysis of Zhong *et al.* [54], *DEFB1*-G1654A may be a genetic risk factor for the development of periodontitis.

It is noteworthy that Vardar-Sengul *et al.* [48] suggested that the expression of hDB1 varies in different periodontal conditions, since comparing gingivitis and aggressive periodontitis, the expression of hBD1 was higher in chronic periodontitis. When the type of periodontitis was considered for data analysis in the meta-analysis of Chen *et al.* [7], the results revealed a significant association between the rs1799946 and rs1800972 polymorphisms and the risk of aggressive periodontitis, but not of chronic periodontitis.

The literature demonstrates an association between caries experience and polymorphisms in the *DEFB1* gene [21, 32, 33, 36, 50]. Interestingly, most of these studies clearly suggest that 2 polymorphisms (rs11362 and rs1799946) in the promoter region, similar to those evaluated in the present study, may be involved in caries susceptibility. Although caries and gingivitis are considered biofilm-dependent diseases, other etiological factors specific to each situation, as well as the evolutionary course

are different, which may partially justify the disagreement with our findings.

Another important aspect is that differences in the characteristics of the studied populations may reflect conflicting results in the literature. The heterogeneous genetic effects on periodontitis risk between the overall analysis and the analysis stratified by ethnicity may be explained, in part, by interactions between genes in a different genetic background, different allele frequencies in races, and interactions between genes and the environment [7]. Additionally, the sample size, differences in experimental design, environmental interactions, age-dependent effects and insufficient statistical power also lead to inconsistencies. The etiological heterogeneity of gingivitis and periodontal disease, regulated by genetic and genetic-environmental control, should also be highlighted as a possible factor for discrepancies between studies [33].

It is noteworthy that the present was the first study to investigate the association of genetic polymorphisms with the presence of gingivitis. In addition, in the present study, the sample consisted of 27 children with a mean age of 9.92, also showing the innovation and originality.

Thus, further studies are needed with a larger number of patients to substantiate the potential functional effects of the rs1799946 and rs11362 reference polymorphisms, as well as other polymorphisms, in the *DEFB1* gene on gingivitis, particularly in children, including the influence on the expression levels of beta-defensins in the oral epithelium.

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