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Influence of polymorphisms in osteoprotegerin on susceptibility to periodontal disease in patients with type 2 diabetes

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Abstract

Objective: The aim of this study was to investigate polymorphisms in the promoter region (-163A/G, -245T/G and -950T/C) of the gene osteoprotegerin (OPG) as well as the distribution and influence of these polymorphisms in patients with type 2 diabetes and periodontitis in comparison to a health control group.

Methods: The study involved the participation of 67 subjects: 32 in the test group (diabetic patients with periodontitis) and 35 in the control group (non-diabetic subjects without periodontitis). Pocket depth, bleeding on probing and attachment loss were assessed for the diagnosis of periodontitis. Six sites on each tooth were probed. Periodontitis was diagnosed when two or more sites had a pocket depth and attachment loss ≥ 4 mm. DNA was obtained from the blood of the subjects for the investigation of OPG polymorphisms using the Restriction Fragment Length Polymorphism (RFLP) method.

Results: No association was found between periodontitis and polymorphisms in the promoter region of the OPG gene in test group ($P>0.005$). The most frequent allele in this group was A163 (81.2%), followed by T245 (75.0%) and T950 (54.7%). The T950 allele, which is a possible marker of osteoclastogenesis, was not associated with periodontal status in patients with diabetes ($P>0.005$).

Conclusion: No association was found between periodontiitis and genetic polymorphisms in the OPG gene in patients with diabetes.

Keywords: Diabetes; periodontitis; osteoprotegerin; polymorphism

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Influência do polimorfismo na osteoprotegerina na suscetibilidade para doença periodontal em paciente com diabetes tipo 2

Resumo

Objetivo: Este estudo analisou a presença de polimorfismos na região promotora -163A/G, -245T/G e -950T/C do gene da osteoprotegerina (OPG), bem como sua distribuição e associação em pacientes diabéticos e com periodontite, comparados ao grupo controle saudável.

Métodos: participaram da pesquisa 67 indivíduos distribuídos em um grupo teste (n=32), constituído por pacientes diabéticos e com periodontite, e outro controle (n=35) com pacientes não diabéticos e sem periodontite. Para o diagnóstico da periodontite, avaliou-se clinicamente profundidade de sondagem, sangramento à sondagem e nível de inserção clínica, sendo sondados seis sítios em cada dente presente e diagnosticada a periodontite na presença de dois ou mais sítios com profundidade de sondagem e nível de inserção clínica ≥4mm. O DNA para a investigação dos polimorfismos foi obtido a partir de amostras sanguíneas dos participantes, o polimorfismo foi avaliado através do método Restriction Fragment Length Polymorphism (RFLP).

Resultados: Não foi observada associação entre polimorfismos da região promotora da OPG no grupo teste ($P>0,005$). O alelo mais freqüente neste grupo foi o A163(81,2%), seguido pelo T245(75,0%) e pelo T950(54,7%). O alelo T950, possível marcador da osteoclastogênese, não foi associado à condição periodontal dos pacientes diabéticos ($P>0,005$).

Conclusão: Não foi encontrada associação entre polimorfismos genéticos da OPG em pacientes diabéticos e com periodontite.

Palavras-chave: Diabetes; periodontite; osteoprotegerina; polimorfismo

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Introduction

Evidence suggests that diabetes mellitus affects periodontal tissue by reducing leukocyte functions, such as chemotaxis, adherence and phagocytosis. Diabetes is also considered a risk factor for periodontal disease, considering the incidence, progression and severity of this condition in diabetic individuals [1-3].

A number of mechanisms may explain the increased susceptibility to periodontitis among individuals with diabetes, in whom sustained hyperglycemia exerts an indirect influence on the cellular level through the production of advanced glycation end products and may also directly affect the cells involved in periodontal homeostasis. Among such cellular alterations, the increased production of matrix metalloproteinases, reduction in collagen synthesis, reduction in growth factors and deregulation of the balance between pro-inflammatory and anti-inflammatory cytokines as well as the balance between mediators of bone metabolism, such as receptor activator of nuclear factor kappa B (RANK), its ligand RANKL and osteoprotegerin (OPG), may predispose individuals with diabetes to a greater incidence and progression of periodontal disease [4]. Indeed, periodontitis is considered the sixth most common complication of diabetes mellitus, according to the American Academy of Diabetes [2,4].

Alveolar bone loss, which is mediated by the immune response of the host to the buildup of bacterial plaque, is one of the main indicators of periodontitis [4]. The assessment of clinical attachment loss and periodontal pockets is often used for the diagnosis of this condition [5,6]. However, new diagnostic modalities for the assessment of the onset and progression of periodontitis are also being employed, such as concentrations of RANK, RANKL and OPG [5-7]. OPG, which is also known as osteoclastogenesis inhibition factor, is a bone-regulating protein made up of five exons that has the capacity to inhibit bone resorption [8,9] and plays an important role in the vascular and immune system [10]. This protein interacts with RANKL, blocking its bonding to RANK, which are proteins responsible for the differentiation and activation of osteoclasts [8,9].

Impairment to bone formation/repair is an important sign of periodontal disease. Under physiological conditions, there is a balance between bone resorption and formation. This balance maintains bone integrity and the metabolism of calcium and is mediated by the RANK/RANKL/OPG system [11]. In certain inflammatory conditions, such as periodontitis, this system is disturbed, causing a reduction in OPG level and an increase in the concentration of RANKL resulting in pathologic bone resorption [11,12].

Differences in the proportions of RANKL/OPG may be related to the severity of periodontal disease [1,5,7]. The importance of the regulation of these proteins in bone destruction has been demonstrated in a number of studies, which state that an increase in RANKL concentration and reduction in OPG concentration may indicate periodontitis [13-15]. Studies report high concentrations of OPG in the plasma and saliva of patients with diabetes, while these concentrations are reduced in individuals with both diabetes and periodontitis [1,5,7]. Moreover, another study reports that high levels of OPG in the gingival tissue of non-diabetic individuals with periodontitis and the negative regulation of these levels in systemically and periodontally compromised individuals may indicate the importance of diabetes mellitus in favoring osteoclastogenesis [15].

Given the importance of the RANK/RANKL/OPG system in the pathogenesis of periodontal disease and its influence on diabetes, the notion that genetic polymorphisms in the OPG gene are related to an increased susceptibility to periodontitis in diabetic patients has been questioned. Twelve different polymorphisms in the OPG gene, including the promoter region corresponding to positions -163A/G, -245T/G and -950T/C, was identified [16]. The identification of these polymorphisms is reported to be a valuable diagnostic tool for assessing the risk of periodontitis [19].

While the literature has demonstrated the effect of the biological activity of a variety of cytokines, such as OPG, on the destruction of the periodontium in individuals with diabetes, the mechanisms involved in this process have not been fully clarified. Thus, the aim of the present study was to analyze the influence of genetic polymorphisms in the OPG gene in individuals with both diabetes and periodontitis.

Materials and methods

Sample

An experimental trial was carried out involving a convenience sample made up of 67 participants: 32 individuals with a diagnosis of type 2 diabetes for at least five years and periodontitis (test group) and 35 non-diabetic individuals without periodontitis (control group). The diagnosis of type 2 diabetes was confirmed based on glucose levels greater than 125 mg/dL and glycated hemoglobin greater than 6.5%. The participants were examined at the Ermírio de Moraes Medical Center and Stomatology Clinic of the Federal University of Pernambuco in the city of Recife, Brazil. This study received approval from the Human Research Ethics Committee of the university (process number: 088/09).

The inclusion criteria were age between 21 and 55 years, either gender and having a minimum of 15 teeth [5,7]. The following were the exclusion criteria: smoking habits; pregnancy or lactation; use of antibiotics; having undergone periodontal treatment in the previous six months; medical treatment for systemic conditions other than diabetes; major complication of type 2 diabetes (retinopathy, neuropathy, nephropathy and atherosclerosis); and menopause.

Clinical parameters

The diagnosis of periodontal disease was made on the basis of clinical parameters, such as probing pocket depth, assessment of clinical attachment loss and bleeding on

probing. Six sites on each tooth were examined using a University of North Carolina millimeter probe (PCPUNC 15® Hu-Friedy). Peridontitis was defined as pocket depth and attachment loss ≥4 mm in two or more sites [7] based on the criteria established by the American Academy of Periodontology.

Polymerase chain reaction

DNA was extracted from blood samples using the DNA blood mini kit (Qiagem), following the manufacturer's instructions. The following primers and annealing temperatures were used: F- AACTTGAACACTTGGCCCTGA and R- AAATTGGACTGCCTGGGG at 60° C for -163A/G and -245T/G; and F- GTTCCTCAGCCCGGTGGCTTTT and R- TGTGGTCCCCGGAAACTTCAGG at 64° C for -950T/C.

Genotypes from the promoter region of the OPG gene were amplified using the conventional polymerase chain reaction (PCR) method in a total volume of 25 µL, containing 12.5 µL of Top Taq Polymerase (Qiagen, Hilden, Germany), 0.6 µL of each primer and 4 µL of DNA. The amplifications were performed with 35 cycles of denaturation at 96° C for 45 seconds, annealing temperatures for each primer (described above) for 45 seconds, extension at 72° C for 45 seconds, with initial denaturation at 96° C for 5 minutes and final extension at 72° C for 10 minutes [17].

The restriction fragment length polymorphism method (PCR-RFLP) was used to detect the length of the amplified fragments following digestion by the restriction enzyme. This method involves a transition base for the A163G genotype (A/G); the T245G genotype (T/G) and T950C genotype (T/C) of the OPG gene, establishing a specific restriction site for each enzyme. RFLP was performed in a total volume of 20 µL, using 1 U of each restriction enzyme (*HinfI, Vspl* and *HincII*) (Promega) and 8 µL of the PCR product, digested at 37° C for 2 hours. The digested products were separated in 3% agarose gel.

Statistical analysis

The frequency of the genotypes and alleles in the test and control groups was analyzed using Pearson's chi-square test (χ^2) and Fisher's exact test. The Kruskall-Wallis test and F test (ANOVA) with Tukey comparisons were used to determine associations between genotypes and clinical parameters. Results achieving a *P*-value<0.05 were considered significant. The statistical calculations were performed using the Statistical Package for the Social Sciences (SPSS, version 15).

Results

Table 1 displays the clinical parameters (probing pocket depth, bleeding on probing and assessment of clinical attachment loss) and age of the participants in both groups. The mean values of each variable were significantly higher in the test group than the control group (*P*<0.001).

The PCR analysis revealed mutations in the -245T/G and -950T/C regions. These mutations were represented by two bands in the respective genotypes, characterizing a (GG) T245G and (CC) T950C homozygous carrier. However, no statistically significant differences between groups were detected with regard to these mutations (*P*>0.05). The greatest variation occurred in the T245G genotype (*P*=0.850) (Table 2). Likewise, no statistically significant association was found between periodontal disease and diabetes with regard to the distribution of alleles (Table 3).

The clinical parameters were analyzed in relation to the polymorphic regions in order to determine possible associations between the severity of periodontal disease and polymorphisms in the OPG gene in patients with type 2 diabetes. In the control group, mean probing pocket depth was lesser in the GG genotype (mutant) and greater in the TT genotype (wild) of the -245T/G region, whereas mean probing pocket depth was lesser in the TT genotype (wild) and greater in the occurrence of the TC or CC genotype (mutant) of the -950T/C region (Table 4). Bleeding on probing was not influenced by the genotype profile of OPG in either the test group or control group. With regard to clinical attachment loss, a significant result was only found in the -245T/G region in the control group, for which the GG mutant genotype achieved the highest mean value.

Table 1. Periodontal status of the study population.

Variable	Group		*P*-value
	Test	Control	
	Mean±SD (Median) [1]	Mean±SD (Median)	
Age (years)	49.34±4.94 (50.00)	28.29±10.99 (23.00)	*P* [2]<0.001*
Probing pocket depth	6.09±1.96 (5.25)	2.47±0.55 (2.50)	*P* [2]<0.001*
Bleeding on probing	38.23±13.27 (34.67)	12.38±3.43 (11.06)	*P* [2]<0.001*
Clinical attachment loss	2.97±0.69 (2.87)	0.71±0.26 (0.69)	*P* [2]<0.001*

* Significant difference to 5.0%.
[1] SD – standard deviation.
[2] Student's t-test with unequal variances.
Source: patients treated at Ermírio de Moraes Medical Center and Stomatology Clinic of Federal University of Pernambuco, Brazil.

Table 2. Occurrence of genotypes according to polymorphic region.

Variable	Group				*P*-value
	Test		Control		
	n	%	N	%	
Total	32	100.0	35	100.0	
Genotype: A163G					
AA	20	62.5	22	62.9	$P^{(1)}=0.976$
AG	12	37.5	13	37.1	
GG	–	–	–	–	
Genotype: T245G					
TT	19	59.4	22	62.9	$P^{(2)}=0.850$
TG	12	37.5	11	31.4	
GG	1	3.1	2	5.7	
Genotype: T950C					
TT	10	31.2	11	31.4	$P^{(2)}=1.000$
TC	15	46.9	17	48.6	
CC	7	21.9	7	20.0	

(1) Pearson's chi-square test.
(2) Fisher's exact test.
Source: patients treated at Ermírio de Moraes Medical Center and Stomatology Clinic of Federal University of Pernambuco, Brazil.

Table 3. Frequency of alleles of osteoprotegerin gene according to polymorphic region.

Variable	Group				*P*-value
	Test		Control		
	n	%	n	%	
Total	64	100.0	70	100.0	
Genotype: A163G					
A	52	81.2	57	81.4	$P^{(1)}=0.979$
G	12	18.8	13	18.6	
Genotype: T245G					
T	48	75.0	55	78.6	$P^{(1)}=0.624$
G	16	25.0	15	21.4	
Genotype: T950C					
T	35	54.7	39	55.7	$P^{(1)}=0.905$
C	29	45.3	31	44.3	

(1) Pearson's chi-square test.
Source: patients treated at Ermírio de Moraes Medical Center and Stomatology Clinic of Federal University of Pernambuco, Brazil

Table 4. Clinical parameters according to polymorphic region in test and control groups.

Genotypes	Clinical Parameters					
	PPD (Mean±SD)		BP (Mean±SD)		CAL (Mean±SD)	
	GT	GP	GT	GP	GT	GC
A163G						
AA	6.10±1.96	2.48±0.50	39.48±12.73	12.93±3.71	2.97±0.68	0.70±0.26
GG	6.08±2.05	2.46±0.66	36.15±14.44	11.43±2.78	2.98±0.72	0.71±0.25
P-Value	$P^{(2)}=0.982$	$P^{(2)}=0.937$	$P^{(2)}=0.502$	$P^{(2)}=0.216$	$P^{(2)}=0.955$	$P^{(2)}=0.930$
T245G						
TT	5.80±1.77	$2.68\pm0.45^{(A)}$	36.84±13.15	11.61± 2.59	2.88±0.62	$0.62\pm0.24^{(A)}$
TG	5.96±1.92	$2.18\pm0.56^{(A)}$	36.52±11.31	14.26±4.47	2.94±0.71	$0.83\pm0.22^{(B)}$
GG	9.50±0.71	$1.75\pm0.35^{(B)}$	60.99±1.75	10.44±0.88	4.05±0.23	$1.00\pm0.18^{(B)}$
P-Value	$P^{(3)}=0.123$	$P^{(3)}=0.010$*	$P^{(3)}=0.101$	$P^{(3)}=0.182$	$P^{(3)}=0.148$	$P^{(3)}=0.024$*
T950C						
TT	6.75±2.25	$2.09\pm0.58^{(A)}$	39.95±13.02	11.58±2.88	3.17±0.78	0.85±0.25
TC	5.97±2.08	$2.62\pm0.45^{(B)}$	40.89±14.14	12.31±3.40	2.93±0.73	0.64±0.24
CC	5.43±0.93	$2.71\pm0.49^{(B)}$	30.06±9.39	13.77±4.32	2.78±0.39	0.63±0.22
P-Value	$P^{(4)}=0.382$	$P^{(4)}=0.017$*	$P^{(4)}=0.183$	$P^{(4)}=0.432$	$P^{(4)}=0.499$	$P^{(4)}=0.058$

* Significant difference to 5.0%.
(1) SD – standard deviation.
(2) Student's t-test with unequal variances.
(3) Kruskal-Wallis test.
(4) F test (ANOVA) with Tukey comparisons.
Source: patients treated at Ermírio de Moraes Medical Center and Stomatology Clinic of *Universidade Federal de Pernambuco*, Brazil.

Discussion

As OPG is involved in the bone remodeling process, polymorphisms in its genetic structure may have a strong impact on the structural and functional properties of this protein and alter the proportions of RANKL/OPG, which would predispose individuals to greater destruction of the alveolar bone [9].

The present study found no influence of polymorphisms in the promoter region of the OPG gene on chronic periodontitis in patients with diabetes. However, further studies with methodological standardization should be carried out to investigate the effect of such polymorphisms in individuals with both diabetes and periodontal disease.

Despite the high prevalence and incidence of periodontal disease in patients with diabetes, few studies have been conducted for the identification of genetic markers for these conditions. Moreover, no studies were found in the literature consulted regarding the influence of genetic polymorphisms in individuals with both diabetes and periodontal disease. It is therefore important to conduct investigations into the association between such polymorphisms and the occurrence of these two diseases, given their bi-directional relationship. Patients with diabetes may have difficulties with regard to bone remodeling, suggesting that bone formation defects could influence the progression of periodontitis in these individuals [7]. Considering the modulating role of diabetes in individuals with chronic periodontitis, it has been suggested that the disease may exert such an effect through the inhibition of OPG production, thereby favoring the bone resorption process over bone formation [1]. Another study [15] found that systemically healthy individuals with periodontitis have high levels of OPG in the gingival tissue in contrast to both systemically and periodontally compromised individuals. However, other experiments [5,10] suggest that patients with both diabetes and periodontal disease have high concentrations of OPG in the saliva and crevicular fluid, which may be related to the vascular involvement that commonly stems from diabetes. Thus, as OPG plays an important role in both bone remodeling and in the immune and vascular systems, high concentrations of OPG may represent a defense mechanism against arterial calcification and other forms of vascular impairment. Moreover, peripheral vascular failure in diabetic patients impairs healing and produces physiological changes that decrease the capacity of the immune system, thereby increasing susceptibility to infections [20].

Despite the bi-directional relationship between diabetes and periodontal disease, to our knowledge, there are few reports in the literature on the association of this relationship with the occurrence of genetic polymorphisms, particularly in OPG. Indeed, studies have only investigated the association between such polymorphisms and periodontal disease [9,17,21]. Based on knowledge that both diabetes and periodontal disease are related to individual susceptibility, it is important to study genetic abnormalities in specific biological markers (mediators of bone metabolism) that may be related to the development of these diseases.

The results of the present study corroborate findings described in previous studies [17,18], which found no association between the -950T/C region and periodontitis. This region has also been assessed in patients with both periodontitis and chronic kidney disease and no association was found [18]. Likewise, no association between the -950T/C region and periodontitis has been found [9]; however, the authors studied polymorphisms in the exons of the OPG gene, whereas the present study focused on the promoter region of the gene.

The results of the present study corroborate findings described by other authors [17,21], who found no association between periodontitis and polymorphisms in the -245T/G promoter region. In the present study, the individuals in the control group exhibited mutations in this region (GG). When associated with pocket depth and clinical attachment loss, such mutations proved significant, as a lesser mean probing pocket depth (P=0.010) and greater clinical attachment loss (P=0.024) were found in the mutant group (GG) of the polymorphic region. However, these associations may have occurred due to the small number of individuals who displayed such mutations, thereby reflecting statistical variation. Besides the small sample size found in the present investigation and previous studies [5,7,10,17], the lack of standardization in the few studies carried out to investigate OPG polymorphisms in patients with type 2 diabetes and periodontal disease underscores the need for further research into this topic.

While there is no consensus in the literature, a number of studies suggest an association between the T950 allele and an increase in osteoclast activity. A greater frequency of this allele in patients with periodontitis has been reported [17]; however, the authors studied genetically homozygous Korean subjects, in contrast to the present sample, which was genetically heterozygous. Another study involved a genetically heterozygous population and found no association between the T950 allele and chronic periodontitis [18], which is in agreement with the findings of the present study.

Associations between OPG polymorphisms and other systemic diseases have often been studied, such as osteoporosis [22] and the T950 allele [23], kidney disease and the -950T/C region [18], vascular disease [20] and menopause and cardiovascular disease and the C950 allele [24,25]. The latter association should be investigated further, as periodontitis may also be considered an important risk factor for cardiovascular disease, which is also associated with diabetes.

Based on the results of the present study, no influence was found regarding polymorphisms in the OPG gene that may explain the association between periodontitis and diabetes. However, further studies should be carried out in order to clarify the role of OPG and other mediators of bone metabolism in the pathogenesis of periodontitis, considering the high incidence, progression and severity of this disease in patients with diabetes.

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