Receptor activator of nuclear factor kappa-B gene polymorphisms in Iranian periodontitis and peri-implantitis patients

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Purpose: Peri-implantitis and periodontitis are inflammatory and infectious diseases of implant and tooth-supporting tissues. Recently, the role of gene polymorphisms of immune response components in the relevant pathogenesis has been investigated. The present study was the first to evaluate the relationship between two known single nucleotide polymorphisms (SNPs) of the receptor activator of nuclear factor kappa-β (*RANK*) gene (rs3018362 and rs35211496) in chronic periodontitis and peri-implantitis patients in an Iranian population.
Methods: Eighty-one periodontally healthy patients, 38 patients with peri-implantitis, and 74 patients with chronic periodontitis were enrolled in this study. DNA was extracted from blood arm vein samples by using Miller's salting out technique according to the manufacturer's instructions given in the extraction kit. The concentration of DNA samples was measured using a spectrophotometer. The genetic polymorphisms of the *RANK* gene were evaluated using a competitive allele specific polymerase chain reaction (KBioscience allele specific PCR) technique. Differences in the frequencies of genotypes and alleles in the diseased and healthy groups were analyzed using chi-squared statistical tests ($P<0.05$).
Results: Analysis of rs35211496 revealed statistically significant differences in the expression of the TT, TC, and CC genotypes among the three groups ($P=0.00$). No statistically significant difference was detected in this respect between the control group and the chronic periodontitis group. The expression of the GG, GA, and AA genotypes and allele frequencies (rs3018362) showed no statistically significant difference among the three groups ($P=0.21$).
Conclusions: The results of this study indicate that the CC genotype of the rs35211496 RANK gene polymorphism was significantly associated with peri-implantitis and may be considered a genetic determinant for peri-implantitis, but this needs to be confirmed by further studies in other populations.

Keywords: Genetic polymorphism; Peri-implantitis; Periodontitis; Receptor activator of nuclear factor-kappa B.

INTRODUCTION

Periodontitis is an infectious disease of the tooth-supporting tissues. Specific periodontal pathogens trigger inflammatory responses and result in progressive bone loss, which eventually leads to exfoliation of the teeth. At present, dental implants are successfully used to replace extracted teeth. Despite the long-term predictability of implants, some complications may occur involving the implant-supporting tissues. Two prevalent complications are peri-implant mucositis and peri-implantitis [1]. Genetic differences are responsible for vari-

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ations in individual immune responses to periodontal pathogens [2].

Receptor activator of nuclear factor kappa-β ligand (RANKL), RANK, and osteoprotegerin (OPG) are members of the tumor necrosis factor (TNF) super family. The RANK/RANKL/OPG signaling pathway is essential for the process of osteoclastogenesis. In humans, RANK is coded by the *TNFRSF11A* gene, which is located on chromosome 18q21 [3]. RANKL and OPG are two competitive ligands for RANK. Under inflammatory conditions, the production of RANKL increases and the RANKL/RANK ratio becomes greater than the OPG/RANK ratio, resulting in increased proliferation of osteoclasts and a higher rate of bone loss. Therefore, RANK-L and OPG regulate bone remodeling via positive or negative stimulation of RANK [4]. In dentistry, evaluation of the roles of RANK, RANKL, and OPG in alveolar bone resorption (seen in periodontitis and peri-implantitis) is a new topic of research [5-7]. Sarlati et al. [8] compared the level of soluble RANKL (sRANKL) in peri-implant cervicular fluid (PICF) in three groups: (1) healthy implants, (2) implants with peri-implant mucositis, and (3) implants with peri-implantitis. The concentration of sRANKL was also assessed to compare the plaque index, probing depth, gingival index, and bleeding on probing around each implant. In the most recent study published by Guncu et al. [9], the concentration of biomarkers associated with osteoclastogenesis in peri-implantitis patients was investigated and compared with the biomarker levels in the peri-implant fluid of healthy controls. The researchers concluded that an unbalanced secretion of biomarkers (e.g., RANKL and OPG) can result in the development of inflammatory peri-implant lesions.

Recently, single nucleotide polymorphisms (SNPs) in the encoding genes of bone remodeling agents (RANK/RANKL/OPG) have been reported in association with bone diseases such as low bone mineral density (BMD), Paget's disease, and osteoporosis [10-13]. SNPs may affect the gene expression or function of proteins [14]. In fact, functional SNPs such as rs35211496 and rs3018362 in the *RANK* gene can change the function of a protein in the translation process.

On the basis of the bone resorption seen in bone diseases (e.g., osteoporosis) and periodontitis/peri-implantitis, and the relationship between the *RANK* gene polymorphisms and bone resorption [10-13], we supposed that the RANK SNP is a genetic determinant for periodontitis and peri-implantitis in some populations. The purpose of the present study was to evaluate the relationship between two functional known SNPs of the *RANK* gene in chronic periodontitis and peri-implantitis in an Iranian population. We analyzed rs3018362 and rs35211496 due to their significant effects on bone resorption in Paget's disease and osteoporosis [10-13].

MATERIALS AND METHODS

A total of 193 individuals were evaluated in this case-control study: 74 patients with chronic periodontitis, 38 patients with peri-implantitis, and 81 patients with healthy periodontal/peri-implant tissues. Understudy subjects were selected among patients presenting to the Department of Periodontology, School of Dentistry, Shahid Beheshti University of Medical Sciences. Demographic characteristics and clinical data of the patients were collected using a checklist. Written informed consent was obtained from all individuals prior to the initiation of the study. The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (89-01-92-7274).

The inclusion criteria were based on both radiographic and clinical parameters including the plaque index, probing pocket depth (PPD > 4 mm), clinical attachment level (CAL ≥ 3 mm), and bleeding index at four sites around the tooth or implant [15]. Periodontitis subjects had to have at least three teeth with bleeding on probing, PPD of > 4 mm, and CAL of ≥ 3 mm in two quadrants (excluding the third molars).

The exclusion criteria were as follows: hepatitis, human immunodeficiency virus infection, chemotherapy, history of systemic/local diseases affecting the immune system or bone metabolism, diabetes mellitus, pregnancy/nursing, diseases other than dental caries and periodontitis, smoking, current orthodontic treatment, and non-Iranian ethnicity.

The inclusion criteria for the peri-implantitis group were no history of periodontitis and presence of one or more implants with a minimum loading period of 12 months. Diagnosis of peri-implantitis was made on the basis of PPD of > 5 mm (with/without suppuration) and radiographic signs of crestal bone loss in at least one area around an implant [16]. Using the implant standard index (ISI) classification, we included patients with ISI VI, VII, and VIII in this study [17], resulting in the consideration of factors such as a 2-mm radiographic bone loss, exposure of at least two threads of implants, and PPD of > 4 mm.

The control group (healthy periodontium) consisted of subjects with no history or clinical signs of periodontitis, no PPD of > 4 mm around any implant/tooth, no radiographic sign of bone resorption, and no loss of clinical attachment around the tooth.

In order to perform genotyping, 5 mL of blood was obtained from an arm vein of each subject and transferred into falcon tubes containing ethylenediaminetetraacetic acid. A unique code was assigned to every subject in order to conduct the laboratory stages blindly. DNA was extracted using Miller's salting out technique according to the manufacturer's instructions given in the extraction kit (Cinnagen Inc., Teheran, Iran). The concentration of DNA samples was measured using a spectrophotometer (75 ngr/μL).

The samples were then transferred into 96 division plates and transported to KBioscience Institute in the United Kingdom. The genetic polymorphisms of the *RANK* gene were evaluated by KBioscience allele-specific polymerase chain reaction technique.

Details of this genotyping can be found on the institute's website at: www.kbioscience.co.uk/reagents/KASP.html. The National Center for Biotechnology Information (NCBI) sequence of the aforementioned alleles is as follows:

rs3018362

GATCATCTTACCTACACCAGGTTCAC[A/G]TTTTCCATCTTAGAGTTATA

Table 1. Demographic and clinical characteristics of understudy groups.

Group	Age (year)	Female	Male	Probing pocket depth (mm)	Attachment/bone loss[a] (mm)
Chronic periodontitis (n=74)	48.3 (26–60)	34	40	5.94±0.67	5.44±0.85
Healthy periodontium (n=81)	38.4 (31–55)	40	41	1.84±0.58	0.17±0.17
Peri-implantitis (n=38)	50.2 (29–60)	18	20	6.86±0.58	4.44±1.69

Values are presented as mean (range) or mean±standard deviation.
[a]Bone loss is related to implants and attachment loss to teeth.

Table 2. Genotype and allele frequencies of the RANK (rs35211496) polymorphism in control and diseased groups.

Group	Genotypes			*P*-value	Alleles		*P*-value
	TT	TC	CC		T	C	
Healthy periodontium	1 (50.0)	22 (40.0)	58 (42.6)	0.000	24 (40.6)	138 (42.2)	0.158
Periodontitis	1 (50.0)	26 (47.3)	47 (35.4)		28 (47.5)	120 (36.7)	
Peri-implantitis*	0 (0)	7 (12.7)	31 (22.8)		7 (11.9)	69 (21.1)	
Total	2 (100)	55 (100)	136 (100)		59 (100)	327 (100)	

Values are presented as number (%).
*$P<0.05$.

Table 3. Genotype and allele frequencies of RANK (rs3018362) polymorphism in control and diseased groups.

Group	Genotypes			*P*-value	Alleles		*P*-value
	AA	GA	GG		A	G	
Healthy periodontium	13 (50.0)	32 (42.1)	37 (40.7)	0.210	58 (45.3)	106 (41.1)	0.169
Periodontitis	7 (26.9)	27 (35.5)	40 (44.0)		41 (32.0)	107 (41.5)	
Peri-implantitis	6 (23.1)	17 (22.4)	14 (15.3)		29 (22.7)	45 (17.4)	
Total	26 (100)	76 (100)	91 (100)		128 (100)	258 (100)	

Values are presented as number (%).

CAGGA
rs35211496
AGTGCGCGCCGGGCCTGGGCGCCCAG[C/T]ACCCGTGTACGGGTTG
GATGTGTGC

IBM SPSS ver. 19.0 (IBM Co., Armonk, NY, USA) was used for the statistical analysis. Differences in the expression of genotypes and the frequency of alleles were assessed between the case and the control groups by using a chi-squared test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

This case-control study was conducted on 193 subjects including 38 patients with peri-implantitis, 81 healthy controls, and 74 chronic periodontitis patients. No significant differences were found among the groups in terms of sex or age. Demographic and clinical characteristics of the three groups are presented in Table 1.

The genotype and allele frequencies of RANK polymorphisms are shown in Tables 2 and 3. An analysis of rs35211496 revealed statistically significant differences in the expression of TT, TC, and CC genotypes ($P=0.00$) (Table 2). No statistically significant difference was detected in this respect between the control group and the chronic periodontitis group.

Table 2 shows a comparison of genotype frequency between the healthy controls and peri-implantitis patients, which revealed a statistically significant difference between them ($P=0.00$). The comparison of genotype frequencies between chronic periodontitis and peri-implantitis groups is demonstrated in Table 2, indicating a significant difference between them ($P=0.00$).

The comparison of the frequency of allele expression in the three groups revealed no significant difference (Table 2), but the expression of the C allele was higher in the control group.

Table 3 shows the genotype and allele frequencies of the rs3018362 polymorphism. The expression of the GG, GA, and AA genotypes and allele frequencies showed no statistically significant difference among the three groups ($P=0.21$).

DISCUSSION

There are multiple risk factors that increase the likelihood of periodontitis and peri-implantitis, such as pathogenic bacteria, tooth deposits, some systemic disorders (e.g., diabetes), smoking,

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and genetic factors. Evidence indicates that genetic differences among individuals may explain why some patients develop periodontal disease and peri-implantitis in the presence of bacteria while others do not. This study was conducted to evaluate the *RANK* gene polymorphisms in chronic periodontitis and peri-implantitis patients compared with healthy subjects for the first time in dentistry. Our results indicate that the rs35211496 gene polymorphism may contribute to the development of peri-implantitis in at least the Iranian population.

The results of this study indicated that the *RANK* gene polymorphism (rs35211496) correlated with peri-implantitis but not with periodontitis. This finding can be justified as follows: In our previous study, we showed that the most prevalent bacteria in Iranians with peri-implantitis were *Porphyromonas gingivalis*, while in periodontitis patients, the most prevalent bacteria were *Tannerella forsythia* [18]. Furthermore, chronic periodontitis and peri-implantitis have histopathological differences. The apical progression of the lesion is more pronounced in peri-implantitis. Additionally, in chronic periodontitis, we can see a self-limiting procedure, but not in peri-implantitis due to the lack of periodontal ligament (PDL) around the implant. Recently, a study concluded that PDL is not only a supporting tissue but also an antibacterial agent against *P. gingivalis* [19]. Thus, the structural differences between the tooth and the implant (particularly PDL) on one hand and the bacterial differences on the other hand help us to better interpret the present study results.

The noteworthy point in the evaluation of the TNF superfamily (RANK/RANKL/OPG) is the important role played by RANK, RANKL, and OPG in bone remodeling. It has been shown that PDL cells in humans have the potential to produce RANKL and OPG; this might explain the elevated levels of RANKL/OPG in the gingival cervicular fluid of patients with periodontitis [7]. Several studies have investigated the relationship between gene polymorphisms of immune system components and periodontal disease in different populations. To the best of our knowledge, no study has assessed the relationship of chronic periodontitis and peri-implantitis with RANK gene polymorphism.

In 2006, Wohlfahrt et al. [20] compared several parameters of SNPs between healthy subjects (n = 82) and severe chronic periodontitis patients (n = 137) in 9 important molecules, including the OPG (245 T > G) level, in a North American white population. They concluded that smoking and male gender increased the disease risk. They also found that there was no significant difference in terms of the frequency of alleles or genotype expression between cases and controls. In 2007, Wagner et al. [6] studied the relationship of gene polymorphisms in interleukin (IL) 1 alpha (–889 C/T), IL-1 beta (+3953 C/T), and OPG (Lys3Asn and Met256Val) with chronic periodontitis in a Caucasian population. Although they found that IL-1 alpha and beta SNPs were associated with the disease, their results failed to prove a statistically significant difference in the genotype frequency of OPG SNPs between the 97 healthy controls and the 97 chronic periodontitis patients.

In 2008, Park et al. [21] investigated the distribution of 8 OPG gene polymorphisms among periodontitis patients (14 aggressive and 35 chronic) and healthy controls (n = 49) in a Korean population. Their results demonstrated that T950C and G1181C were statistically more common in periodontitis cases. They also found that G1181C is strongly associated with aggressive periodontitis. Consequently, they concluded that the TG haplotype of OPG gene polymorphisms could be considered a genetic determinant of periodontitis. In a meta-analysis study by Lee et al. [22] conducted in 2010, a correlation was detected between the GG genotype in the G1181C OPG polymorphism and the reduction in BMD, while there was no relationship between the T950c polymorphism and the BMD.

To the best of our knowledge, there is no published article regarding the evaluation of the correlation between periodontitis or peri-implantitis, and the RANK SNP. However, there is some evidence supporting the relationship between the RANK SNP and bone disease [23,24].

The soluble RANKL and OPG ratio in the fluid around an intraosseous implant was assessed by Arikan et al. [7] in 2008. RANK levels were not related to the age of the patients or other clinical parameters, but the OPG level was related to the gingival index, bleeding on probing, and PICF values.

In 2010, Sarlati et al. [8] investigated the RANKL levels in the peri-implant cervicular fluid. Forty patients receiving implants were divided into clinically healthy, peri-implant mucositis, and peri-implantitis groups on the basis of the clinical and radiographic findings. Filter paper strips were used to collect PICF for 30 seconds in the base of the pocket. PICF samples were obtained from buccal and lingual aspects of implants. The plaque index, probing depth, gingival index, and bleeding on probing were recorded at six sites around the implant. There were no statistically significant differences in the sRANKL level between the healthy control, peri-implant mucositis, and peri-implantitis groups. Also, no statistically significant difference was detected between the sRANKL level and the clinical parameters.

In 2011, Fan et al. [25] studied the RANKL and OPG expressions in chronic apical periodontitis patients and healthy controls in a Chinese population. The RANKL levels and the RANKL/OPG ratio in chronic apical periodontitis patients were significantly higher than in healthy periapical tissues. In contrast, there was no relationship between the RANKL/OPG ratio and inflammatory cell infiltration. Kadkhodazadeh et al. [26] showed that the *RANKL* gene polymorphism plays a critical role in Iranian patients with peri-implantitis.

To summarize, our results indicated that *RANK* gene polymorphism (rs35211496) is associated with peri-implantitis. This finding is a step forward, but further studies are required to clearly illuminate the relationship of specific gene polymorphisms with chronic periodontitis and peri-implantitis for the following reasons: First, a limited number of individuals participated in the study. Second, periodontitis and peri-implantitis are multifactorial diseases, and they are not affected by only one gene. The third reason is that other polymorphisms of this gene should be considered. We pro-

pose that this issue be investigated in a larger sample of Iranian patients and in different populations. Multiple peri-implantitis cases should also be compared with single peri-implantitis cases. Finally, due to the highly genetic nature of aggressive periodontitis, it is recommended that these SNPs be evaluated in aggressive periodontitis, too. The results of this study indicate that the CC genotype of the rs35211496 *RANK* gene polymorphism was significantly associated with peri-implantitis and may be considered a genetic determinant for peri-implantitis, but this has to be confirmed by further research including randomized clinical control studies.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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