Short Communication

The association between selected RANKL gene polymorphisms and chronic/aggressive periodontitis in Iranian subjects

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Abstract
The primary aim of this study was to investigate the status of RANKL6-7 gene polymorphism in patients with chronic (mild, moderate, severe) and aggressive periodontitis as well as healthy controls. We examined 80 patients for the RANKL6-7 polymorphisms (rs1054016 and rs9567000). Polymorphism was determined by polymerase chain reaction (PCR) followed by direct sequencing. No statistically significant association was found between the polymorphism in the RANKL 7 gene and periodontal disease (P<0.55). There was also no polymorphic allele observed in RANKL 6 gene of the study population. We found no association between the studied RANKL polymorphisms and chronic/aggressive periodontitis.
Keywords: Periodontitis; Single nucleotide polymorphism; Genetics; RANKL

Periodontitis has been defined as "an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss and bone loss (Flemmig, 1999). The primary factors for periodontitis are bacteria, however, the extent and severity of periodontal lesions can be influenced by environmental factors, acquired diseases, and genetic predisposition (Hart and Kornman, 1997; Oliver *et al*., 1991). Clinicians have long known that susceptibility to periodontitis differs among racial and ethnic groups (Oliver *et al*., 1991). In one of the earliest studies in 1966 Trott and Cross reported that "certain individuals are more at risk for periodontitis than others" (Trott and Cross, 1966). Twenty years later in a classic study Löe *et al*. showed that "among individuals with poor oral hygiene and no access to dental care, some developed disease at a rapid rate whereas others experienced little or no disease" (Loe *et al*., 1986). Again twenty years later Van der Velden *et al*. (2006) in a study on the initiation and progression of periodontal breakdown suggested that "not everybody is equally susceptible to periodontitis" (Van der Velden *et al*., 2006). Heritable risk factors may be related to inflammatory or immune mechanisms that could modify the pathogenic potential of bacterial plaque in susceptible individuals (Shenkein and Van Dyke, 1994).

The basis for the association of the cytokine gene polymorphism and periodontal disease is that carriage of certain alleles of a cytokine gene is related to increased production of a given cytokine. The risk of

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having periodontal disease has been shown to be related to carriage of rare alleles of single cytokines such as IL-1, TNFα, and IL-6 (Yoshie *et al*., 2007a; Yoshie *et al*., 2007b; Takashiba and Naruishi, 2006; Loos *et al*., 2005; Shapira *et al*., 2005; Taylor *et al*., 2004).

Receptor activator of nuclear factor-kappa B ligand (RANKL) and its receptor RANK have been recognized as key factors regulating osteoclast formation (Takayanagi *et al*., 2000). RANKL a membrane-bound or soluble protein belonging to the tumor necrosis factor (TNF) superfamily that is primarily produced in osteoblastic lineages and activated T cells. RANKL stimulates osteoclast differentiation and activation, and inhibits osteoclast apoptosis. Binding of RANKL to RANK expressed on the surfaces of osteoclasts and their precursors, promotes osteoclast differentiation and activation (Yasuda *et al*., 1998a). Osteoprotegerin (OPG), a soluble TNF receptor–like molecule, is the inhibitor of osteoclast differentiation. It binds to RANKL and blocks RANKL from interacting with RANK (Yoshie *et al*., 2007; Yasuda *et al*., 1998b).

A positive association in the genetic markers of RANK and OPG has been observed in some diseases such as Paget's disease (Wuyts *et al*., 2001), human osteoporosis (Ohori *et al*., 2002), and familial expansile osteolysis (Hughes *et al*., 2000). There were also reports suggesting OPG gene polymorphism, alone and in combination with the RANKL polymorphism as a genetic factor associated with lumbar spine and femoral neck bone mineral density, vascular calcification and bone metabolism in humans (Kim *et al*., 2007; Rhee *et al*., 2010). Although significant differences in the distribution of specific polymorphisms in the OPG gene has been observed by some investigators, between periodontitis patients and normal individuals (Park *et al*., 2008). Some others found no association between OPG polymorphisms and chronic periodontitis (Baioni *et al*., 2008) as well as RANK/RANKL/OPG polymorphisms in the pathogenesis of aggressive periodontitis (Soedarsono *et al*., 2006).

Therefore the aim of this study was to investigate the distribution of RANKL6-7 gene polymorphisms in patients with chronic (mild, moderate and severe) and aggressive periodontitis as well as healthy controls. Our second aim was to investigate if any relationship existed between periodontal disease status and polymorphisms in the studied genes.

A total of 80 Iranian individuals (38 women and 42 men; age range: 20-59 years) were enrolled in the study from 2009 to 2010. Fifteen patients with mild chronic periodontitis, 15 patients with moderate chronic periodontitis, 15 patients with severe chronic periodontitis and 18 patients with aggressive periodontitis were recruited at the Department of Periodontics, Dental Branch, Islamic Azad University. Subjects without periodontal disease (N= 17) were included as healthy matched controls representing normal population which consisted of university staff and students who were informed of the study and volunteered to give their blood samples to be used as reference samples. All the individuals were examined by the same periodontist. Voluntary informed patient consents were obtained from all the patients and the study protocol was approved by the ethical and research committee of Dental Branch, Islamic Azad University according to the declaration of Helsinki (version 2002). Exclusion criteria were: Immunosuppressive chemotherapy, oral diseases other than caries and periodontal diseases, use of orthodontic appliances, present necrotizing ulcerative gingivitis and periodontitis, HIV infection, a history of systemic or local diseases with influence on the immune system, diabetes mellitus, antibiotic therapy during the past 4 months and current pregnancy and lactation.

The clinical diagnosis of the patients was based on American Academy of Periodontology criteria (Armitage, 1999). The diagnostic criteria of periodontitis were established on the basis of radiographic and clinical parameters. The examiner was trained prior to the study. Periodontal examination included: Probing Pocket Depth (PPD), Clinical Attachment Level (CAL), and Plaque Index (PLI). Probing Pocket Depth was recorded for all teeth at four sites per tooth (midmesial, midbuccal, middistal and midlingual) using a Williams probe (Hufriedy®). The probe was inserted into each sulcus/pocket using gentle pressure until it could not be advanced further apically. The probe penetrated the coronal level of the junctional epithelium and caused no pain. Clinical attachment level was recorded from the cementoenamel junction (CEJ) to the base of the sulcus/pocket for all teeth at the four mentioned sites. The presence of dental plaque was recorded following the O'Leary Plaque Index using a disclosing tablet (0=absence and 1=presence of bacterial plaque) (O'leary *et al*., 1972). Subjects were assigned to clinical categories with regard to their clinical attachment level (CAL). The control group was older than 35 years and had no clinical attachment loss in any teeth. Subjects older than 35 years with CAL≥5 mm in at least three teeth in at least two quadrants were considered as having severe chronic periodontitis

and subjects older than 35 years with CAL = 3 or 4 mm in at least three teeth in at least two quadrants were considered as having moderate chronic periodontitis. Subjects older than 35 years with CAL = 1 or 2 mm in at least three teeth in at least two quadrants were considered as having mild chronic periodontitis. Subjects who had attachment loss of ≥ 4 mm on at least two permanent teeth, including at least one first molar with age of onset of periodontitis < 35 years were considered as having aggressive periodontitis. Alveolar bone loss in all the aggressive periodontitis patients and chronic periodontitis ones was assessed using full Mouth radiographs. Data regarding smoking was obtained by interviewing the subjects in association with the clinical examination.

Venous blood (1 ml) was taken by standard venipuncture from each individual and mixed with 1 mg/ml EDTA. Genomic DNA was extracted from blood using the nucleon genomic DNA extraction kit (Macherey- Nagel Co., Duren, Germany) according to the manufacturer's instructions. The resulting DNA samples were stored at -70ºC in TE Buffer 10 mM tris, 1 m/M EDTA) until required. The following allelic variants were investigated: RANKL 7 (rs1054016, exon 5, chromosome 13, G/T) and RANKL 6 (rs9567000, exon 5, chromosome 13, C/T). Sequence references were obtained from The National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with accession number NT-024524. Table 1 provides information about the candidate SNPs tested.

Oligonucleotide primers specific for the human RANKL 6 and RANKL 7 genes, were designed on the basis of published sequence information from the GenBank database. Polymarase chain reaction (PCR) amplification was performed by using PCR primer sets designed to amplify coding exon of the candidate RANKL genes. A constant reverse primer was used for sequencing. The reaction medium of 25 µl contained 12.5 µl PCR Master mix (CinnaGen Co.,Tehran, IR Iran), 1 µl template (genomic DNA), 2 µl of each primers and 7.5 µl water. The PCR amplification was performed for 40 cycles, as follows: at 95ºC for 5 minutes for denaturation, at 58ºC for 10 S for annealing, and at 72ºC for 1 min for extension. PCR primer sets and sequencing primers are shown in Table 2.

PCR products were purified using column-based method. After purification, the amplified genomic DNA was sequenced (Sanger Sequencing Method) by using ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA,USA) at Macrogen Inc., Seoul, Korea. In order to determine the validity of the method, 20 samples were analyzed twice and the results were found identical. The associations of allele frequencies in patients and healthy controls were analyzed by the chi-square test. P-values ≤ 0.05 were considered to be statistically significant.

The characteristics of patients and healthy controls are described in Table 3. There was only one smoker among our patients. Table 4 shows RANKL 6 and RANKL 7 allele frequencies in patients and controls. No statistically significant association was found between the polymorphism in the RANKL 7 gene and periodontal disease (P<0.55). There was also no polymorphic allele observed in RANKL 6 gene of the

Table 1. Candidate SNPs* characteristics.

Gene Symbol	dbSNP number	Chromosome location in NCBI build 37.2	Substitution Major>minor	location
RANKL** 6	9567000	13:43181437	C>T	Exon 5
RANKL 7	1054016	13:43182002	G>T	Exon 5

*Single Nucleotide Polymorphism. ** Receptor Activator of Nuclear Factor –kappa B ligand.

Table 2. Oligonucleotide sequence of RANKL* for polymerase chain reaction (PCR) amplification and sequencing.

Gene	Fragment ID	Forward primer	Reverse primer	Direction
RANKL6	5	TGAAGGATCATCTGAAGGGGC	GCT GAA GTG GAG AGG GTG TCA TC	Reverse
RANKL7	5	TGCCGCAAATTGTACCTTTTTTT	GCT GAA GTG GAG AGG GTG TCA TC	Reverse

*Receptor Activator of Nuclear Factor –kappa B ligand.

Table 3. Characteristics of patients and healthy controls.

Characteristics	Mild CP* patients (N=15)	Moderate CP patients (N=15)	Severe CP patients (N=15)	Aggressive periodontitis patients (N=18)	Healthy controls (N=17)
Age	34±8.6	37±7.6	47±6.2	34.3±12.6	35.6±9.8
Gender					
male	6	9	6	7	10
female	9	6	9	11	7
PPD**	3.22±0.55	4.05±0.94	6±1.89	6.8±1.36	1.5±0.53
CAL***	1.67±0.48	3.56±0.51	6.65±1.78	6±1.12	0
PI****	62.66±18.15	75.16±16.39	90.83±11.14	38.33±28.43	25±7.90

*Chronic periodontitis, **Probing pocket depth, ***Clinical attachment level, ****Plaque Index.

Table 4. Allele frequency of the RANKL*7 SNP**:

SNP Alleles	Mild CP *** patients (N=15)	Moderate CP patients (N=15)	Severe CP patients (N=15)	Aggressive periodontitis patients (N=18)	Healthy controls (N=17)	Chi-squared
T	10	8	6	9	11	χ^2 =3.03
G	5	7	9	9	6	P=0.55

*Receptor Activator of Nuclear Factor- kappa B ligand, **Single Nucleotide Polymorphism, ***Chronic Periodontitis.

study population. In the present study we investigated the association of RANKL gene polymorphisms with periodontitis and found no association between the study RANKL polymorphisms (rs1054016 and rs9567000) and chronic/aggressive periodontitis.

After the introduction of a new classification for the periodontal disease by the American Academy of Periodontology (1999), periodontal diagnoses are now based primarily on the rate of disease progression. Periodontitis is now considered to have three primary forms: chronic, aggressive and as a manifestation of systemic diseases (Armitage, 1999). Aggressive periodontitis is a specific type of periodontal disease that is characterized by rapid attachment loss and bone loss resulting in tooth loss at early onset (<35 years of age). Although most studies showed that genetic factors influence the susceptibility to aggressive periodontitis, there are studies reporting that not only aggressive periodontitis but also chronic periodontitis may have a genetic background.

The relationship between gene polymorphism and periodontal disease expression has long been studied in several investigations by comparing the frequencies of alleles or genotypes between subjects with various degrees of periodontal disease or between periodontitis patients and reference subjects. The basis for the association of cytokine gene polymorphism and periodontitis is that some alleles of a cytokine gene are related to increased production of a given cytokine. Genetic polymorphisms arise as a result of gene mutations. All organisms undergo spontaneous mutations as a result of normal cellular function or random interactions with the environment. An alteration that changes only a single base pair is called a point mutation. Not all point mutations are repaired and can therefore be transmitted by inheritance through generations. The variation at the site harboring point mutations has recently been termed a "single nucleotide polymorphism" (SNP). SNPs represent the most common form of DNA variation in the human genome, and polymorphic alleles have been implicated in the increased susceptibility to complex human diseases such as periodontitis (Baylor *et al*., 2004).

Several studies have indicated variation in cytokine levels among patients suffering from periodontitis and that this variation may be related to individual susceptibility (Mcfarlane *et al*., 1990; Kjeldsen *et al*., 1995). Some studies have reported a role for interleukin-1 gene polymorphism in the risk assessment for chronic periodontitis (McDevitt *et al*., 2000; Gore *et al*., 1998; Kornman *et al*., 1977). The risk of having periodontal disease has also been related to carriage of the rare alleles of cytokines such as TNF-α, IL-6 and CD14. Trevonen *et al*. (2007) reported that

the CD14^{-260} and the IL-6^{-174} polymorphisms associated with the severity of periodontal disease after controlling for significant confounding factors.

To our knowledge, there is only one study (Soedarsono *et al*., 2006) evaluating the association of RANKL gene polymorphisms with aggressive periodontitis (Soedarsono *et al*., 2006). They reported that SNPs identified in the RANKL/RANK/OPG genes have no significant association with aggressive periodontitis in the Japanese population. On the other hand a number of polymorphisms in the RANKL gene have been described in previous studies and their association with "Bone Mineral Density" and "Aortic Calcification" has been reported in a Korean population (Rhee *et al*., 2010; Kim *et al*., 2007).

In the present study we have tried to investigate the association of these polymorphic alleles with both chronic and aggressive periodontitis as well as healthy controls. Aggressive periodontitis is characterized by 3 major common features: Noncontributory medical history, Rapid attachment loss and bone destruction and Familial aggregation of cases. It is proposed that chronic periodontitis progresses in episodes of exacerbation and remission. Socransky *et al*. (1984) termed this the "burst hypothesis" of disease progression. Clinically the nature of the disease can only be confirmed by repeated examinations over time. We have chosen our subjects at a single visit. As reported by steer *et al*. in 2003 using clinical measures alone to define disease may not be appropriate for genetic studies (Steer and Macgregor, 2003). Although in the present study all subjects (patients and healthy controls) were chosen from the same public dental center, in order to obtain equivalence between the groups.

In the present study, the lack of differences in the prevalence of the investigated polymorphisms between chronic/aggressive periodontitis and healthy controls may be related to the small sample size. A lack of microbial data is also a shortcoming of this study. We focused on single nucleotide polymorphisms (SNPs) because they are common in the human genome and can be typed in a relatively inexpensive manner.

Common polymorphisms are those with minor allele frequencies >20%. We chose two common polymorphisms (RANKL6 and RANKL7) since they were both located on exon 5 of chromosome 13 and can be typed easily. These findings do not preclude the possibility of other allelic variants in the RANKL gene being associated with the pathogenesis of periodontitis. Since genetic polymorphisms vary in different ethnic populations, conclusions about disease association cannot be extended to other populations.

We found no association between the studied RANKL polymorphisms and chronic/aggressive periodontitis.

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