

ANALYSIS OF IL-6, IL-10 AND NF-KB GENE POLYMORPHISMS IN AGGRESSIVE AND CHRONIC PERIODONTITIS

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SUMMARY

Objective: Pro-inflammatory cytokines, interleukin-6 (IL-6), demonstrated to be suppressed by interleukin-10 (IL-10) are known to be regulated by the transcription factor nuclear factor- κ B (NF- κ B). The aim of this study was to ascertain the association between genetic polymorphism of these genes (IL-6(-174), IL-10(-597) and NF- κ B1-94ins/del) and chronic/aggressive periodontitis.

Methods: Forty-five patients with chronic periodontitis (CP), 58 patients with aggressive periodontitis (AP) and 38 periodontally healthy subjects were included in this study. Genomic DNA was isolated from whole blood samples. The NF- κ B, IL-6, and IL-10 polymorphisms were determined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Results: Among subjects for the ins/ins genotypes of NF- κ B1 gene, the AA genotypes of IL-10 presented a higher frequency in chronic periodontitis group than in healthy controls ($p=0.023$). A statistically significant difference in genotyping frequencies between AP group and healthy controls was observed for the IL-6 gene. The AA genotype of IL-10 was overrepresented in CP and AP groups compared to healthy controls (OR=9.93, 95% CI: 2.11–46.7, OR=5.7, 95% CI: 1.22–26.89, respectively).

Conclusions: Within the limits of this study, it can be concluded that the IL-10 (-597) AA genotype is associated with susceptibility to chronic/aggressive periodontitis and IL-6 (-174) GG genotypes and G allele seems to be associated with aggressive periodontitis.

Clinical relevance: The results of the current study indicate that IL-6 and IL-10 genotypes seem to be associated with aggressive periodontitis. Also, the AA genotypes of IL-10 presented a higher frequency in chronic periodontitis subjects with carrying NF- κ B1 ins/ins genotypes.

Key words: periodontitis, polymorphism, NF- κ B, IL-6, IL-10

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INTRODUCTION

Periodontitis is an inflammatory disease associated with a bacterial infection. Immune response of the periodontal tissues to infection is influenced by environmental factors as well as genetic factors (1). The complex cytokine network that mediates the immune response includes pro-inflammatory cytokines, anti-inflammatory cytokines and specific cytokine receptors. Furthermore, genetic variants of some cytokines confer susceptibility to periodontitis (2–4).

Nuclear factor- κ B (NF- κ B) is a transcription factor that is involved in inflammation, cell survival, angiogenesis and apoptosis (5). Five members of the NF- κ B family have been identified, and they are NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), RelA (p65), RelB and c-Rel. Several polymorphisms have been identified in the promoter region of NF- κ B1 gene, which is insertion/deletion (-94ins/delATTG) (6). However, one study conducted on periodontal disease reported that del/del genotype of NF- κ B1 gene was associated with aggressive periodontitis (7). NF- κ B is highly activated at sites of inflammation and can induce transcription

of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6 and IL-8), chemokines, adhesion molecules, and matrix metalloproteinases (8). Also, some cytokines can directly activate NF- κ B pathway, thus establishing a positive auto-regulatory loop that can amplify the inflammatory response and increase the duration of chronic inflammation. However, NF- κ B, can act as an anti-inflammatory factor and has a role in IL-10 gene expression (9).

Among several kinds of cytokines, IL-6 has been shown to increase in the gingival crevicular fluids and in the gingival tissues of individuals with periodontitis (10). IL-6 is also a potent stimulator of osteoclast differentiation and bone resorption and an inhibitor of bone formation (2). In the IL-6 gene, there are more than four known promoter polymorphisms: -597 (G/A), -572 (C/G), -174 (G/C) single nucleotide polymorphisms, and -373 AnTm polymorphisms (11). As the -174 G/C and -572 C/G polymorphisms of the IL-6 gene increase IL-6 expression, they may be associated with susceptibility to periodontitis (2, 12, 13). However, the frequency of many genetic alleles varies among ethnic groups, several studies have found contradictory results regarding the relationship between this gene and periodontitis (2, 10, 14).

IL-10, an anti-inflammatory cytokine, plays a role in periodontitis by inhibiting synthesis of pro-inflammatory cytokines such as IL-1, -2, -6 and stimulating protective antibody production (15). The gene encoding for IL-10 is mapped on chromosome one (1q31-32) (16). A large number of single nucleotide polymorphisms have been identified in the IL-10 gene promoter (17). Of interest are the polymorphisms at -1082 (-1087), -819 (-824) and -592 (-597) (18). However, the presence of an association between IL-10 gene polymorphisms and clinical manifestations of chronic and aggressive periodontitis is controversial. Some of the studies failed to find any evidence of association (4, 19, 20) and others revealed a possible association between this polymorphism and periodontitis (17, 21–24). Considering the multifactorial nature of a periodontal disease, little is known among the complex interaction of the polymorphisms in the promoter regions of IL-6 (-174), IL-10 (-597) and NF- κ B1 (-94ins/del) genes and clinical parameters of periodontitis. Therefore, we carried out a clinical study to assess the effect of these polymorphisms in chronic and aggressive periodontitis and to evaluate associations among the polymorphisms themselves.

MATERIALS AND METHODS

Study Population

The study had a case-control design. This study presented the gene polymorphisms part of collected data at approved studies by the Medical Ethics Committee of Cumhuriyet University (2011/031 and 2012-02/35). All subjects signed a consent form. A total of 141 subjects, including 45 with chronic periodontitis, 58 with aggressive periodontitis, and 38 periodontally healthy individuals were selected for the study from the Department of Periodontology, Faculty of Dentistry, Cumhuriyet University. All subjects were Turkish Caucasian and have low to moderate socioeconomic status. Excluding criteria were diseases of oral soft and hard tissues in oral cavity, excluding caries and periodontitis; presence of orthodontic apparatuses in mouth; usage of systemic antibiotics in period of three months before engagement in this study; pregnancy and lactation; diabetes; appliance of immune-suppressive therapy; and current or former smokers.

The diagnosis of subjects was established on the basis of clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Diseases and Conditions (25).

Generalized Chronic Periodontitis Group

The generalized chronic periodontitis group consisted of 45 subjects. The diagnosis of chronic periodontitis was assigned to patients when they demonstrated clinical attachment levels (CAL) in at least 30% of their teeth (CAL \geq 5 mm) with pocket depth $>$ 5 mm.

Generalized Aggressive Periodontitis Group

The generalized aggressive periodontitis group consisted of 58 subjects with a history of rapid attachment loss and bone destruction. The diagnosis of aggressive periodontitis was assigned

to patients when they demonstrated pocket depth (PD) and CAL \geq 5 mm and radiographic bone loss of \geq 30% of root length on at least three teeth other than first molars or incisors. In addition, patients were not included if it was assumed that periodontal destruction was caused by local risk factors like poor restorations and poor oral hygiene.

Healthy Control Group

Control subjects were designated as healthy if they had full mouth probing pocket depth less than 3 mm, gingival index scores equal to 0 and there was no radiographic evidence of alveolar bone loss. These individuals were systemically and periodontally healthy volunteers.

Clinical Measurements

A clinical examination was performed by one calibrated examiner (E.P.G.). Full-mouth measures of probing pocket depth (PD), clinical attachment levels (CAL) were obtained at six points per tooth. The presence of supragingival plaque was scored using plaque index (PI) (26). Gingival inflammation was scored using gingival index (GI) (26). Bleeding on probing (BOP) was also recorded. PD and CAL measures were obtained using a Williams' periodontal probe.

DNA Extraction and Genotyping

From each patient, we collected 2 ml of blood from the antecubital vein. The blood samples were collected in sodium EDTA vacutainers, and stored at -80°C until genetic analyses were performed. The extraction of DNA was performed with a commercial kit (Invitrogen, Cambrillo, USA) following the manufacturer's protocol.

Each sample of DNA was analyzed for polymorphisms in the IL-6 gene at position -174, in the IL-10 gene at position -597 and in the NF- κ B1 gene at position -94 ins/del ATTG with PCR-RFLP method. Primer sequences are shown for each primer pair in Table 1.

DNA samples were amplified by PCR (PTC-200 thermal cycler, MJ Research, Watertown, MA, USA) with specific primers for each examined polymorphism. PCR reaction mixtures (50 μ l) contained 5 μ l \times 10 (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 0.8% Nonidet P-40) PCR buffer, 0.2 mM each dNTP, 10 pmol of each primer (forward and reverse control and allele specific), 1.5 mM MgCl_2 , 200 ng of genomic DNA (Fermentas, Maryland, USA), 0.5 U Taq DNA polymerase (Fermentas, Maryland, USA) and each internal control primers.

For interleukin-6 (-174G/C), the following PCR protocol was used: 94°C for 3 min (initial denaturation), 94°C for 30 s (denaturation), 62.6°C for 40 s (annealing), 72°C for 40 s (extension). The total number of cycles was 35 followed by a final extension of 72°C for 5 min. We used NlaIII and a cleavage protocol following the manufacturer's instructions. NlaIII cleaves a restriction site at position -174 when G is present, leading to fragments of 29, 176, 202, and 233 base pairs (bp) in length or 29, 111, 122, 176 and 202 bp when C is present. In heterozygote individuals (A/C) have 42, 66, 232, 240 and 306 base pairs in length.

Table 1. Primer sequences and reaction properties for genes

Marker	Primer sequences	Annealing temperature (°C)	Base pairs of amplicon size
IL-6 (-174)	5'-GGGCTGCGATGGAGTCAGAG-3'	62.6	639
	5'-TCCCTCACACAGGGCTCGAC-3'		
IL-10 (-597)	5'-ATCCAAGACAACACTACTAA-3'	48	412
	5'-TAAATATCCTCAAAGTTCC-3'		
NF-κB1 (-94ATTG)	5'-TGGGCACAAGTCGTTTATGA-3'	51.5	285
	5'-CTGGAGCCGGTAGGGAAG-3'		

For interleukin-10(-597A/C), the following PCR protocol was used: 94 °C for 3 min (initial denaturation), 94 °C for 30 s (denaturation), 48 °C for 40 s (annealing), 72 °C for 40 s (extension). The total number of cycles was 35 followed by a final extension of 72 °C for 5 min. We used RsaI and a cleavage protocol following the manufacturer's instructions. RsaI cleaves a restriction site at position -597 when A is present, leading to fragments of 42, 66, 232 and 240 base pairs (bp) in length or 42, 232 and 306 bp when C is present. In heterozygote individuals (A/C) have 42, 66, 232, 240 and 306 base pairs in length.

For NF-κB (-94ATTG), the following PCR protocol was used: 94 °C for 3 min (initial denaturation), 94 °C for 30 s (denaturation), 51.5 °C for 40 s (annealing), 72 °C for 40 s (extension). The total number of cycles was 35 followed by a final extension of 72 °C for 5 min. We used PflM 1 and a cleavage protocol following the manufacturer's instructions.

The alleles were separated by 3% agarose gel electrophoresis and stained with ethidium bromide. Following electrophoresis, the PCR products were visualized under ultraviolet light.

Statistical Analysis

The chi-square test was used to test for deviation of genotype frequencies from Hardy-Weinberg equilibrium and to compare the genotype distributions among individuals with GAgP and healthy controls. Odds ratios (ORs) and 95% confidence intervals were calculated to determine the strength of the association. A p-value of <0.05 was considered significant.

Statistical analysis was performed using the program SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Demographic data and clinical parameters were expressed as a mean ± standard de-

viation. Genotype frequencies were tabulated by direct counting and allele frequencies were calculated from the observed number of genotypes. Chi-square (χ^2) analysis was used to test for deviation of allele frequencies and genotype distribution. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were also calculated for significant associations. Kruskal-Wallis test was used to perform group comparisons. The relationship among all genotypes and chronic/aggressive periodontitis was analyzed by logistic regression analysis while adjusting for potential confounding factors including age and gender. A p value of <0.05 was considered statistically significant. The power of the analysis was completed by the utilizing data from a previous publication (19). An alpha of 0.05 was selected for calculation. The required sample size was 50 in AgP group, giving a statistical power of 80%.

RESULTS

The frequencies of NF-κB1 and IL-10 genotypes in all groups were found in accordance with Hardy-Weinberg equilibrium ($p > 0.05$), whereas IL-6 genotypes showed disequilibrium ($p < 0.05$).

The demographic and clinical characteristics of the study groups are summarized in Table 2. When comparing the groups with the healthy controls, no statistically significant differences in age and gender could be detected.

The genotype distribution fulfilled Hardy-Weinberg criteria. The distribution of all genotypes for study genes among the study groups is presented in Table 3. Distribution of the NF-κB1 gene ins/del, del/del, ins/ins genotypes was not different among the

Table 2. Demographic and clinical characteristic of study groups

	Chronic periodontitis N = 45	Aggressive periodontitis N = 58	Healthy controls N = 38
Age (years)			
Mean ± SD	33.8 ± 6.0	29.9 ± 5.5	32.8 ± 6.4
Range	20–46	20–39	24–46
Gender			
Male, n (%)	13 (28.9)	26 (44.8)	11 (28.9)
Female, n (%)	32 (71.1)	32 (55.2)	27 (71.1)
PD (mm, mean ± SD)	4.4 ± 0.7	5 ± 0.8	1.5 ± 0.3
CAL (mm, mean ± SD)	4.8 ± 0.7	5.4 ± 1	NA

PD – probing depth; CAL – clinical attachment level; SD – standard deviation; NA – not applicable

study groups ($p > 0.05$). Also, the relation of NF- κ B1 genotypes with IL-6 and IL-10 genotypes in patients with chronic/aggressive periodontitis subjects and healthy controls was analyzed. Among subjects with the ins/ins genotypes of NF- κ B1 gene, the AA genotypes of IL-10 presented a higher frequency in chronic periodontitis group than in healthy controls ($p = 0.023$). No statistically significant associations were found between NF- κ B1 and IL-6 polymorphisms among all groups.

A statistically significant difference in genotyping frequencies between AP group and healthy controls was observed for the IL-6 gene ($p = 0.002$). The GG genotypes were associated with increased susceptibility to AP group (GG/GC+CC: OR = 3.54, 95% CI: 1.49–8.42). No difference was found in IL-6 genotype when compared CP group with healthy controls. However, there was a significant difference in the IL-6 genotypes frequency considering all periodontitis patients (CP+AP group) compared to controls, and the calculation of the OR revealed that patients with the GG genotype seemed to be three times susceptible to periodontitis (OR = 3.54, 95% CI: 1.43–6.92).

With regard to the IL-10 gene polymorphism, statistically significant differences in genotyping distribution were observed between AP group and healthy controls and CP group and healthy controls. The AA genotype was overrepresented in CP and AP

groups compared to healthy controls, suggesting that the carriage of the AA genotype was associated with an increased risk of periodontal disease (AA/AC + CC: OR = 9.93, 95% CI: 2.11–46.7, OR = 5.7, 95% CI: 1.22–26.89, respectively). Regarding the IL-10 genotype, significant differences were found in all periodontitis patient (CP + AP groups) compared to healthy controls, and the OR increased to 7.39 (95% CI: 1.67–32.69).

The frequency of G allele at -174 positions of the IL-6 gene were higher in CP, AP and CP+AP groups than those of the healthy controls and these differences were significant. However, there were no significant differences in the distribution of the IL-10-A allele among all groups.

DISCUSSION

In the present study, the associations among three different gene polymorphisms (NF- κ B1, IL-6, and IL-10) in aggressive and chronic periodontitis were investigated. We demonstrated that the -592 polymorphisms in the IL-10 gene are associated with the susceptibility of CP and AP with relevant OR values and association of the ins/ins genotype of NF- κ B1 gene with the AA genotypes of IL-10 observed in chronic periodontitis. Also, the

Table 3. Genotypes and alleles distribution of polymorphisms in the NF- κ B (-94 ins/del), IL-6 (-174) and IL-10 (-597) genes among study groups

Genotypes	All periodontitis	Chronic periodontitis	Aggressive periodontitis	Healthy controls	AP+CP vs. controls p-value	AP vs. controls p-value	CP vs. controls p-value
NF-κB1							
ins/ins	33 (32)	14 (31.1)	19 (63.8)	9 (23.7)	ns	ns	ns
ins/del	62 (60.2)	25 (55.6)	37 (32.8)	26 (68.4)			
del/del	8 (7.8)	6 (13.3)	2 (3.4)	3 (9.5)			
IL-6							
CC	13 (12.6)	8 (17.8)	5 (8.6)	13 (34.2)	0.003	0.004	ns
GC	29 (28.2)	12 (26.7)	17 (29.3)	13 (34.2)			
GG	61 (59.2)	25 (55.6)	36 (62.1)	12 (31.6)			
IL-10							
CC	11 (10.7)	5 (11.1)	6 (10.3)	2 (5.3)	0.003	0.002	0.002
AC	62 (60.2)	24 (35.6)	38 (65.5)	34 (89.5)			
AA	30 (29.1)	16 (53.3)	14 (24.1)	2 (5.3)			
Alleles							
NF-κB1							
Ins	128 (62.1)	53 (58.8)	75 (64.6)	44 (57.8)	ns	ns	ns
Del	78 (37.9)	37 (41.2)	41 (35.4)	32 (42.2)			
IL-6							
C	55 (27.3)	28 (31.1)	27 (23.2)	39 (51.3)	<0.001	0.011	<0.001
G	151 (72.7)	62 (68.9)	89 (76.8)	37 (48.7)			
IL-10							
C	84 (40.7)	34 (37.7)	50 (43.1)	38 (50)	ns	ns	ns
A	122 (59.3)	56 (62.3)	66 (56.9)	38 (50)			

Data given in n (%)
ns – non significant

results of this study indicate that IL-6 GG genotype and G allele appear to have a role in susceptibility to AP.

Human NF- κ B1 gene plays a critical role in coordinating the immune system through its ability to regulate the transcription of a broad variety of genes implicated in the immune system including those of pro-inflammatory and anti-inflammatory cytokines (26). Also, an *in vitro* study suggested that IL-10 inhibits TNF-induced and LPS-induced DNA binding NF- κ B in a dose-dependent manner and specifically affects the p65/p50 heterodimer complex (28). The mechanism behind NF- κ B1 in relation to disease susceptibility remains unclear although -94 del ATTG has been proven to reduce activation of NF- κ B1 transcription. A meta-analysis suggests an association between NF- κ B1 -94ins/delATTG promoter polymorphism to certain autoimmune and inflammatory disease (diabetes mellitus, ulcerative colitis and psoriasis) in Asian population but not in the Caucasian population (6, 27). However, only one study suggested significant association of the homozygous mutation genotype del/del with the occurrence of aggressive periodontitis in a cohort of German Caucasians (7). In our study, we found no association of NF- κ B1 polymorphism with CP and AP. However, as the functional interaction between NF- κ B and other cytokines has been demonstrated, we decided to investigate whether any genotype combination among subjects of the ins/ins genotypes of NF- κ B1 gene, the AA genotypes of IL-10 presented a higher frequency in chronic periodontitis group than in healthy controls.

Our results found no association between NF- κ B1 and IL-6 gene polymorphisms. However, in this study, IL-6 GG genotypes could not modify the risk of chronic periodontitis but increased the risk of aggressive periodontitis. Several studies have been published on whether the IL-6 polymorphism predisposes to periodontitis (29–31). However, the results of studies on the associations between these polymorphism and clinical forms of periodontitis are contradictory (13, 19, 32, 33). In a study (30), that was performed to compare the frequencies of cytokines (CD14, IL-6, TNF- α , IL-10, TLR-4) genotypes in patients with CP, was found that an evident association only exists between T genotypes of CD14 (-260) and the GG genotype of IL-6 (-174), and the extent of periodontal disease. Similar to our findings, the study by Nibali et al. (33), which involved 224 AP patients and 231 controls, revealed that the -174 G allele is significantly associated with an increased risk of AP. Although there are different findings in the literature (2, 10, 34), Fishman et al. (12) suggested that higher serum IL-6 levels were associated with the carriage of allele G for the IL-6 (-174) gene. In our study, with regard to allele distribution, -174 G allele significantly increased in both chronic and aggressive periodontitis.

Allelic variation in cytokine genes regulating their expression results in phenotypic differences in cytokine response (3, 35). The synthesis of monocyte-derived pro-inflammatory cytokines including IL-1, IL-6 and IL-8 are inhibited by IL-10 so that this cytokine down-regulates immune response (24). Therefore, the levels of IL-10 production would appear to be critical in immune regulation. Considering the pro-inflammatory and anti-inflammatory profiles of IL-6 and IL-10, respectively, we evaluated the relation IL-6 with IL-10 genotypes in study groups. No association was observed between these genotypes and the study groups. This was similar to the results of Brazil investigation, in which the TNF- α and IL-10 genotypes did not relate to different clinical forms or severity of periodontitis (20).

Regarding the IL-10 polymorphism, in contrast to other studies (15, 22–24), an association between allele A and either CP or AP was not observed in our study. A recent meta-analysis suggests that the IL-10 -597 A allele can provide an increased CP risk compared with the C allele. However, some studies are in agreement with our results that found no association between the -597 A allele and CP (3, 4, 20). Concerning AP, similar to our findings, the existing studies suggest no association of AP with IL-10 gene polymorphism, except a weak trend in the IL-10 -597 C>A polymorphism (17, 19, 24, 36, 37).

Nevertheless, some limitations of our study should be mentioned. Firstly, we did not measure RNA expression or protein levels of cytokines, and thus we do not know the functional consequences of the polymorphisms of these genes in aggressive and chronic periodontitis. Secondly, we did not assess the other single nucleotide polymorphisms of these genes and the effect of haplotype combinations about IL-6, IL-10, and NF κ B1 genes. A precise statement about a possible association of a genetic locus with a disease can only be made if the full haplotype information had been assessed (38).

Although ethnic factors might play a role in the variability of results in a different population, within the limitation of sample number in this study, IL-6 -174 G allele seems to be associated with CP and AP, but GG genotype associated with only aggressive periodontitis. On the other hand, AA genotype of IL-10 gene contributes to susceptibility to CP and AP and association of the ins/ins genotype of NF- κ B1 gene with the AA genotypes of IL-10 observed in chronic periodontitis. Finally, we suggest that more extensive studies in other ethnical populations should be undertaken in order to analyze associations among these gene polymorphisms in the pathogenesis of periodontitis.

Conflict of Interests

None declared

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