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Interleukin-4 (C-590T) and interferon-γ (G5644A) gene polymorphisms in patients with periodontitis

B. Hooshmand[1], M. Hajilooi[2], A. Rafiei[3], KH. Mani-Kashani[4], R. Ghasemi[5]

[1]Department of Peridontology, School of Dentistry, Hamadan University of Medical Sciences, [2]Department of Immunology, Molecular Medicine Research Center, Hamadan University of Medical Sciences, Hamadan, Iran, [3]Department of Immunology and Microbiology, Sari Medical School, Mazandaran University of Medical Sciences, Sari, Iran, [4]Ward of Community Medicine, Hamadan University of Medical Sciences and [5]General Dentistry. Hamadan University of Medical Sciences, Hamadan, Iran

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Background and Objective: Periodontitis is a multifactorial disease and immunologic and genetic factors have an important role in its pathogenesis. Mutation in the promoter regions of the interleukin-4 and interferon-γ genes has been reported to modify the protein expression. The objective of this study was to evaluate the possible role of interleukin-4 (C-590T) and interferon-γ (G5644A) polymorphisms in the susceptibility to periodontitis.

Material and Methods: In this case–control study, 53 patients (36 women and 17 men), comprising 27 patients with aggressive periodontitis and 26 patients with chronic periodontitis, and 56 healthy volunteers, were enrolled. DNA was isolated from all subjects, and the polymerase chain reaction-sequence specific primer method was used to study the interleukin-4 (C-590T) and interferon-γ (G5644A) gene polymorphisms.

Results: Our results showed no significant difference in the allele and genotype frequencies of interleukin-4 (C-590T) and interferon-γ (G5644A) gene polymorphisms between patients with periodontal disease and controls.

Conclusion: The results suggest that the interleukin-4 (C-590T) and interferon-γ (G5644A) gene polymorphisms may not be associated with the susceptibility of Iranian individuals to periodontitis.

A. Rafiei, Department of Immunology and Microbiology, Sari Medical School, Khazar Boulevard, Sari, Iran
Tel: +98 151 3543088
Fax: +98 151 3543087
e-mail: rafiei1710@gmail.com

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Periodontal disease is probably one of the most common chronic diseases in adults and is initiated by an overgrowth of specific gram-negative bacteria that replace the normal microbiota (1). This may be caused by disequilibrium in the host, which may be a result of several factors, such as modification of the environmental conditions of a proportion of beneficial bacteria producing inhibitory substances and/or by the decreasing efficacy of the host immune system (2). There is no doubt that the balance between local levels of key cytokines, stimulated in response to periodontopathogenetic bacteria and their products, is important in determining the outcome of an immune response to a given pathogen (3).

$CD4^+$ T cells can be distinguished, based on their pattern of cytokine production, into T-helper 1 and T-helper 2. T-helper 1 cells secrete interleukin-2 and interferon-γ, thus promoting cell-mediated immunity, whereas T-helper 2 cells produce interleukin-4, -5, -10 and -13, thereby facilitating humoral immunity (4). The characteristic cytokine products of T-helper 1 and T-helper 2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. Thus, interferon-γ selectively inhibits the proliferation of T-helper 2 cells, whereas interleukin-4 and interleukin-10 inhibit cytokine synthesis by T-helper 1 cells (5,6). Moreover, interleukin-4 suppresses the synthesis of pro-inflammatory cytokines, including interleukin-1 and tumor necrosis factor-α, which induce several events associated with inflammation, tissue destruction, bone

resorption and the production of matrix metalloproteinases and prostaglandin E2 (7).

It has been hypothesized that susceptibility to periodontitis may involve a T-helper 2 response to specific types of periodontal bacteria (8) and that a localized lack of interleukin-4 may increase the predisposition to periodontitis (9). It has recently been demonstrated that the susceptibility to chronic periodontitis correlates with heritability in approximately 50% of cases (10) and it has been postulated that the susceptibility to aggressive periodontitis may also be heritable (11). Polymorphisms in a range of human cytokine genes have been correlated with different levels of protein production. A single nucleotide polymorphism at position −590 in the interleukin-4 gene promoter region was described (12). This polymorphism was shown to increase gene function and is associated with elevated levels of the immunoglobulin E phenotype in asthmatic families (13). As interleukin-4 seems to play a role in periodontal disease, the investigation of genetic polymorphisms that affect its transcriptional activity may provide important information on its function in periodontal disease. Similarly, a G/A polymorphism at position 5644 in the 3′ untranslated region of the interferon-γ (G5644A) gene might affect gene expression (14). The 3′-untranslated region region plays an important role in the expression of many eukaryotic genes by governing mRNA stability, localizing mRNA and regulating translation efficiency, and any polymorphism in this region of the gene might affect gene expression. Few data in the literature refer to frequencies of cytokine-encoding gene polymorphisms in the Iranian population (15) and there is no report of their possible correlations with periodontal disease. In this study we investigated the possible association between genotypes of these two critical cytokines and periodontitis.

Material and methods

Study population

A convenience sample of 106 unrelated nonsmoking subjects was recruited for the study. Fifty-three patients (36 women and 17 men; age range: 15–60 years), consisting of 27 patients with aggressive periodontitis and 26 patients with chronic periodontitis, were enrolled. Periodotitis was diagnosed on the basis of three clinical parameters: probing depth; clinical attachment loss; and bleeding on probing index and radiographic findings (16). None of the patients had a history of current manifestation of systemic diseases. Patients with severe medical disorders (diabetes mellitus, immunological disorders, hepatitis, human immunodeficiency virus infection, and cardiovascular involvement), use of orthodontic appliances, need for premedication for dental treatment, chronic usage of anti-inflammatory drugs, present acute necrotizing ulcerative gingivitis, and woman who were pregnant or lactating, were excluded from the study.

The diagnostic criteria of chronic periodontitis were (a) the amount of periodontal destruction was consistent with the presence of local factors such as plaque and calculus and (b) at least two sites had a probing depth of ≥5 mm and clinical attachment loss of ≥1 mm in every quadrant; fewer than eight teeth showed alveolar bone absorption of more than two-thirds of the root length. The clinical criteria of aggressive periodontitis were the presence of eight or more teeth, at least three of which were other than central incisors or first molars, which had a probing depth of ≥5 mm and clinical attachment loss of ≥3 mm. Subjects, even at young ages, not reporting familial aggregation of the disease, were not classified as aggressive periodontitis cases and were not included in the present study.

In addition, 56 healthy volunteers (28 women and 28 men; age range: 18–48 years), without any periodontal disease, systemic inflammatory disease or surgical treatment in the last 4 wk were considered as controls. The absence of periodontitis was determined according to the following criteria: (a) a minimum of 22 teeth *in situ*; (b) no sites with a probing depth of > 3 mm and (c) the absence of clinical attachment loss. None of the control subjects had a history of periodontitis or tooth loss because of pathogenic tooth mobility. Furthermore, there was no bleeding on probing or radiographic evidence of bone loss in these subjects. All subjects signed an informed consent form before enrollment into the study.

DNA isolation

Ten millilitres of venous blood was collected from each subject into tubes containing 50 mmol/L EDTA, and genomic DNA was isolated from anticoagulated peripheral blood Buffy coat using Miller's salting-out method (17). Then, the genomic DNA was stored at −80°C until required for genotyping. The genotyping was performed using the polymerase chain reaction-sequence specific primer method (18). Internal control primers were included as a control for false-negative reactions. The control primers (5′-TGC CAA GTG GAG CAC CCA A-3′ and 5′-GCA TCT TGC TCT GTG CAG AT-3′) were used at 0.2 μmol/L.

The interleukin-4 polymorphism at position C-590T was identified by the sequence-specific forward primers 5′-CTA AAC TTG GGA GAA CAT TGT C and 5′-CTA AAC TTG GGA GAA CAT TGT T, in combination with the consensus reverse primer, 5′-AGTACAGGTGGCATCTTGG-AAA, with an expected product size of 130 bp. Amplification was carried out using a DNA Technology MTC 400 in a total volume of 15 μmol/L that contained 100 ng of genomic DNA, 1 μmol/L of each allele-specific primer pair, 200 μmol/L of dNTPs, 10 μmol/L Tris-HCl (pH 8.3), 50 mmol KCl, 1.5 mmol/L $MgCl_2$ and 0.5 IU of *Taq* DNA polymerase. The reaction was carried out as follows: initial denaturation at 94°C for 2 min, followed by 10 cycles of amplification at 96°C for 20 s and annealing at 64°C for 50 s, with extraction for 40 s at 72°C, followed by 20 cycles of denaturation at 96°C for 20 s and annealing at 61°C for 50 s, with extraction for 40 s at 72°C. The size of the product was 130 bp. Internal control primers were used for the interferon-γ gene at a concentration of 0.2 μmol/L (5′-TGC CAA GTG GAG

CAC CCA A-3′ and 5′-GCA TCT TGC TCT GTG CAG AT-3′).

The interferon-γ polymorphism at position 5644 (A/G) was identified by the sequence-specific forward primers 5′-CCT TCC TAT TTC CTC CTT CG and 5′-ACC TTC CTA TTT CCT CCT TCA, in combination with the consensus reverse primer, 5′-GTC TAC AAC AGC ACC AGG C, at a final concentration of 1 μmol/L with an expected product size of 298 bp. The amplified polymerase chain reaction products were separated by 2% agarose gel electrophoresis, followed by staining in 0.5 μg/mL ethidium bromide and ultraviolet visualization. The agarose gels were analyzed by an investigator unaware of the origin of the samples.

Data analysis

Allele and genotype frequencies were obtained by direct counting. The distribution of the interleukin-4 or interferon-γ genotype and allele frequencies in control and disease groups was analyzed by the Chi-square test. The study groups were tested for Hardy–Weinberg equilibrium comparing the expected with the observed genotype frequencies. The results of the logistic regression model were expressed as odds ratio and 95% confidence interval. All *p*-values were evaluated in a two-sided model, and a *p*-value of < 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS 10.0 software package (SPSS, Chicago, IL, USA).

Results

The clinical and demographic data of the study populations are shown in Table 1. DNA samples from 53 patients with periodontal disease and from 56 healthy individuals were analyzed for C-590T polymorphism of the interleukin-4 gene and G5644A polymorphism of interferon-γ. The frequencies of these two cytokine genotypes in patients and control individuals were found to be in accordance with those expected by the Hardy–Weinberg equilibrium. The allele and genotype frequencies of the C-590T polymorphism of the interleukin-4 gene were not significantly different between periodontitis (aggressive or chronic combined) patients and controls ($p = 0.07$ and $p = 0.15$, respectively), although the mutant homozygous genotype was slightly more common in patients (Table 2). In order to establish any correlation between the control subjects and a specific periodontitis condition (aggressive or chronic), genotypic comparison was performed between healthy controls and subjects with either aggressive or chronic periodontitis, separately. The genotype frequency distributions of the C-590T polymorphism of the interleukin-4 gene did not differ in a statistically significant manner between controls and either aggressive or chronic periodontitis subjects ($p = 0.21$ and $p = 0.3$, respectively).

In addition, as shown in Table 3, the allele and genotype frequencies of the G5644A polymorphism of the interferon-γ gene were not significantly different between subjects with periodontitis (aggressive or chronic combined) and controls ($p = 0.81$ and $p = 0.19$, respectively), although the heterozygous genotype was slightly more common in the subjects with periodontitis.

However, stratification of patients according to disease condition revealed no significant difference between healthy controls and either aggressive or chronic periodontitis subjects in regard to allele and genotype frequency distributions.

Discussion

This study investigated the frequencies of two single nucleotide polymorphisms (interleukin-4 C-590T and interferon-γ G5644A) in subjects of Iranian origin and studied their correlation with periodontal conditions. The main finding was no significant differ-

Table 2. Allele and genotype frequencies of the C-590T polymorphism of the interleukin-4 gene in patients with periodontal disease and in healthy individuals

	Periodontal disease			
Interleukin-4 polymorphism	All	Chronic periodontitis	Aggressive periodontitis	Controls
Allele: *n*/total (%)				
−590T	21 (19.8)	10 (18.5)	11 (21.1)	34 (30.4)
−590C	85 (80.2)	44 (81.5)	41 (78 9)	78 (69.6)
Genotype: *n*/total (%)				
T-590T	1 (1.9)	1 (3.7)	0	5 (8.9)
T-590C	19 (35.8)	8 (29.6)	11 (42 3)	24 (42.9)
C-590C	33 (62.3)	18 (66.7)	15 (57 7)	27 (48.2)

No statistical differences were observed at any comparison (Chi-square test, $p > 0.05$). The values are the numbers (percentages) of patients or controls positive for each allele or genotype.

Table 1. Clinical and demographic data of patients with periodontal disease and healthy controls

Group	Total number	Men (%)	Women (%)	Age (yr)	Probing depth (mm)	Clinical attachment loss (mm)	Bleeding on probing index (%)
Aggressive periodontitis	27	3 (11.1)	24 (88.9)	42.3 ± 6.7	6.26 ± 1.01	5.07 ± 0.97	88.9 ± 6.26
Chronic periodontitis	26	14 (53.8)	12 (46.2)	47 ± 8.21	5.06 ± 1.37	3.96 ± 1.2	82.96 ± 9.6
Periodontitis (combined)	53	17 (32.1)	36 (67.9)	45.7 ± 5.9	5.67 ± 1.34	4.53 ± 1.2	86 ± 8.5
Healthy controls	56	28 (50)	28 (50)	44.8 ± 2.6	1.62 ± 0.31	0.0	0.0

Table 3. Allele and genotype frequencies of the G5644A polymorphism of interferon-γ gene in patients with periodontal disease and healthy individuals

Interferon-γ polymorphism	Periodontal disease			Controls
	All	Chronic periodontitis	Aggressive periodontitis	
Allele: *n*/total (%)				
5644 A	53 (52)	30 (55.6)	23 (47.9)	60 (53.6)
5644G	49 (48)	24 (44.4)	25 (52.1)	52 (46.4)
Genotype: *n*/total (%)				
A5644A	13 (25.5)	9 (33.3)	4 (16.7)	20 (35.7)
A5644G	27 (52.9)	12 (44.4)	15 (62.5)	20 (35.7)
G5644G	11 (21.6)	6 (22.2)	5 (20.8)	16 (29.6)

No statistical differences were observed at all comparisons (Chi-square test, $p > 0.05$). The values are the numbers (percentages) of patients or controls positive for each allele or genotype.

ence between healthy controls and subjects with periodontitis (aggressive, chronic or combined) concerning any of the investigated polymorphisms.

The cellular mechanisms involved in periodontitis, and the cytokine profiles at the local and peripheral levels, have been described previously (8,19). Subjects susceptible for the progression of periodontitis present a T-helper-2-dominated response, whereas nonsusceptible subjects respond predominantly with a T-helper-1-dominated response. The cytokine profiles of cells extracted from humans with periodontal disease have been analyzed by several investigators. Specifically, interleukin-4 has been analyzed alone or in combination with other cytokines at local and periodontal levels. Similar results have been obtained by investigating the mRNA levels in inflamed gingival tissues (20,21). It has been reported that the concentration of interferon-γ is significantly higher in serum samples and gingival tissue biopsies from periodontitis patients than in healthy controls (22) but the levels of interleukin-4 are generally low or even undetectable. In recent years, the role of genetic risk factors in the pathogenesis of periodontitis has been investigated, showing an association of different genetic polymorphisms with periodontal disease (23–25). Therefore, interleukin-4 and interferon-γ gene polymorphisms might be related to susceptibility to periodontal disease.

The interferon-γ AA genotype at site UTR5644 is associated with low interferon-γ production (26). Despite certainty about the role of interferon-γ in the pathogenesis of periodontal disease, in this study there was surprisingly no significant difference between genotypes of interferon-γ overall or in the disease condition separately. Only a slight increase in the frequency of the heterozygous genotype (intermediate producers) was found among the patients. This finding is agreement with another study, which found no significant difference in the frequency of the interferon-γ G5644A polymorphism between patients with brucellosis and controls (15). This interesting result may be partly a result of the role of ethnicity in the distribution of polymorphic alleles. The lack of association of the interleukin-4 (C-590T) gene polymorphism with susceptibility to periodontitis in Iranian individuals found in our study is in line with other studies that reported no significant association of interleukin-4 polymorphisms with the risk of aggressive periodontitis in Caucasian and Japanese papulations (27).

Although in the current study we did not observe an association between the polymorphic loci examined and periodontal disease, we cannot exclude that other polymorphic variations present within these genes (28), or their receptors, may denote susceptibility to periodontal disease. In this regard, Tanaka *et al.* reported that a polymorphism within the interferon-γ receptor gene may result in a shift to the T-helper 2 response and this shift may increase the susceptibility to systemic lupus erythematosus (29). On the other hand, data deriving from the present and other studies, similar in subject sample and number of investigated genomic markers, should be interpreted with caution. The large-scale approach and family linkage analysis also appear promising and might be meaningful in studying the periodontitis–genetic association.

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