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ORIGINAL ARTICLE

Association of the polymorphisms in promoter and intron regions of the interleukin-4 gene with chronic periodontitis in a Turkish population

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Abstract

Objectives. The etiology of periodontitis is related to the interaction between micro-organisms and host responses. Host modifying factors, such as genetic predisposition, may increase the severity of periodontitis. Recent works have shown that the levels of cytokine expression are regulated by genetic polymorphisms, and that these variations can interfere with progression of the disease. This study therefore aimed to evaluate whether interleukin (IL) 4 gene polymorphisms are associated with severe generalized chronic periodontitis. ***Material and Methods.*** Seventy-five severe generalized chronic periodontitis patients and 73 healthy subjects were examined. Blood samples were taken and genomic DNA was amplified by polymerase chain reaction (PCR). Identification of 70 base-pair repeat polymorphisms in intron 2 and C→T polymorphisms at −590 position of the promoter region was performed through PCR-restriction fragment length polymorphism (RFLP). ***Results.*** No significant differences were found in the allele and genotype frequencies between the control and periodontitis group. ***Conclusion.*** The *IL-4* polymorphisms were not related to severe generalized chronic periodontitis in a Turkish population.

Key Words: *Chronic periodontitis, interleukin-4 gene, promoter and intron polymorphisms*

Introduction

The periodontal diseases are initiated by microbial plaque, which accumulates in the gingival crevice region and induces an inflammatory response. This inflammation, chronic gingivitis, may progress in certain susceptible individuals to the chronic destructive inflammatory condition termed periodontitis [1]. Currently, there are two major forms of periodontitis: chronic (CP) and aggressive (AgP) [2]. The risk for periodontitis is not shared equally by the population. This differential risk for periodontitis is consistent with heritable elements of susceptibility, but direct evidence for a differential genetic contribution to periodontitis comes from several sources [1]. While microbial and other environmental factors are believed to initiate and modulate periodontal disease progression, there is strong supporting evidence that genes play a role in the predisposition to and progression of periodontal diseases [3]. Support for this statement comes from studies of humans and animals indicating that genetic factors influence inflammatory and immune responses in general, and periodontitis in particular [1,4–6]. Individuals may respond differently to common environmental challenges, and this differential response is influenced by the individual's genetic profile [1]. Periodontal lesions contain both T cells and macrophages, which can produce cytokines such as interleukin-4 (*IL-4*), interleukin-1 (*IL-1*) and interleukin-6 (*IL-6*). All these cytokines have been shown to play a part in B-cell activation, proliferation and differentiation [7].

IL-4 is considered an anti-inflammatory cytokine because it possesses important functions in the modulation of B cells [8]. Several studies have shown that *IL-4* downregulates macrophage function [9–11]. *IL-4* inhibits the secretion of prostaglandin E2 (PGE2) and cytokines by macrophages [9,10]. Furthermore, *IL-4* can downregulate the CD14

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receptor [12] and is found to induce apoptosis in monocytes [13]. Alterations in *IL-4* gene may increase disease severity through downregulation of *IL-4* production [8]. The human gene for *IL-4* maps to the long arm (q23–31) of chromosome 5, and is located within a cluster of other cytokine genes [14,15]. *IL-4* is an 18 kDa glycoprotein that inhibits Th1 cells while stimulating a Th2-type of immune response [16].

IL-1 gene polymorphisms were the first described genetic markers related to periodontal disease. A positive correlation between the presence of specific base-pair alterations in the *IL-1* gene and increased risk for CP was demonstrated in Caucasians [17,18]. Another genetic marker, *IL-4*, was correlated with susceptibility to periodontal disease activity in Caucasians [8,11,19].

Several polymorphisms in the *IL-4* gene are known to play roles in diseases such as periodontitis, multiple sclerosis, asthma and atopy, e.g. a 70 bp repeat polymorphism in intron 2 [8,19] and *IL-4* promoter polymorphism (-590 C$\rightarrow$T) [8,19–21]. *IL-4* levels in the sera of periodontitis patients were significantly lower compared with controls, suggesting an association of these polymorphisms with the disease [8].

The primary etiologic factor of CP is the infection caused by gram-negative micro-organisms. However, the influence of genetic factors in the etiology of this inflammatory disease has also been reported [17,22,23]. The aim of the present study was to find the association of *IL-4* polymorphisms with severe generalized CP.

Material and methods

Subjects

Seventy-five patients with severe generalized CP and 73 healthy subjects were included in the study. The mean (SD) age of CP patients (40 F, 35 M) was 47.2 (1.08) years (ranging from 31 to 70 years). The mean (SD) age of healthy controls (37 F, 36 M) was 44.6 (0.92) (ranging from 30 to 65) years. Age and gender of CP and control groups were matched. The patient and healthy controls signed an informed consent form after receiving information about the study. The study protocol and consent forms were approved by the University Institutional Review Board. All the patients fulfilled the diagnostic criteria defined by the International Workshop for a Classification of Periodontal Diseases and Conditions for CP [24]. None of the included subjects had a history or current manifestation of systemic disease (especially cerebrovascular disease, hypertension, hyperlipidemia, obesity, atherosclerosis, and coronary heart disease), diseases of the oral hard or soft tissues except dental caries and periodontal diseases, chronic use of anti-inflammatory drugs, diabetes, hepatitis or HIV infection, immunosuppressive chemotherapy, history of any disease known to severely compromise immune function, smoking, and current pregnancy and lactation.

All subjects were Caucasian, of Turkish origin, and from the northern region of Turkey. In addition, healthy subjects who had no familial relationship to the chronic periodontitis group were included in the study as a control group.

Clinical assessments

The same investigator made clinical assessments of all subjects at their first visit. Prior to actual data collection, 10 subjects were randomly selected and used to calibrate the investigator. The investigator evaluated the subjects on two separate occasions, 48 h apart. Calibration was accepted if measurements at baseline and at 48 h were similar to the millimeter at $>90\%$ level.

The assessed clinical parameters were: probing pocket depth (PPD) and clinical attachment loss (CAL). PPD (the distance in millimeters from the free gingival margin to the bottom of the pocket) and CAL (the distance in millimeters from the cemento-enamel junction to the bottom of the pocket) [25,26] of all the teeth were assessed using a William's Probe (Hu-Friedy, Chicago, Ill., USA) at six sites of a tooth: mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual.

The diagnosis of severe generalized CP was made dependent on the severity of periodontal disease on the basis of the amount of CAL, and according to the extent of disease sites on the basis of the number of sites with PPD ≥ 4 mm involved. Patients exhibiting CAL ≥ 5 mm and $>30\%$ of the sites with PPD ≥ 4 mm were considered with severe generalized CP [27]. The healthy controls comprised unrelated Turkish subjects residing in the same geographic area as the CP patients who did not have a history of periodontitis. These periodontally healthy individuals did not show CAL, PPD >3 mm at more than one site, or radiographic evidence of bone loss [28]. Subjects who did not exhibit the above clinical parameters were excluded from the study.

The mean (SD) values of PPD, CAL, and sites with PPD ≥ 4 mm (%) for patients with CP were 4.68 (0.08), 5.69 (0.10), and 73.29 (1.50), respectively. The mean PPD was 1.79 (0.02) in healthy controls.

DNA extraction and identification of genotype

Genomic DNA was extracted from whole blood treated with EDTA by a standard salting-out method [29]. DNA was dissolved in Tris-EDTA buffer (10 mM Tris pH 7.8, 1 mM EDTA) (Merck, Darmstadt, Deutschland). The final preparation was stored at -20°C until used.

Analysis of the IL-4 70 bp repeat polymorphism in intron 2

The *IL-4* 70 bp repeat polymorphism was determined by a slight modification of the polymerase chain reaction (PCR) described by Mout et al. [30] using the following primers. *IL-4* (RP2A) A: 5′- TAG GCT GAA AGG GGG AAA GC-3′ and *IL-4* (RP2B) B: 5′- CTG TTC ACC TCA ACT GCT CC-3′. Hot start PCR was performed in a Touchgene gradient termalcycler (Techne, Princeton, N.J., USA) in a 25 μl reaction mixture containing 200 ng DNA, 2 mM MgCl, 1.25 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and 25 pmol of each primer (MWG, Ebersberg, Germany). The following cycling conditions were used: an initial denaturation at 95°C for 5 min was followed by 35 cycles at 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1 min (extension) and 72°C for 7 min (final extension). PCR products were visualized on a 2% agarose gel after electrophoresis and ethidium bromide staining. The 183 bp-long product is designated as allele 1 (polymorphic allele) and 254 bp as allele 2 (wild-type allele).

Analysis of the IL-4 promoter polymorphism at −590 (C→T)

The *IL-4* promoter polymorphism was determined by a slight modification of the PCR described by Walley & Cookson [20] using the primers: *IL-4* (AW41A) A: 5′- ACT AGG CCT CAC CTG ATA CG-3′ and IL-4 (AW41B) B: 5′-GTT GTA ATG CAG TCC TCC TG-3′. A hot start PCR was performed as described above. The cycling conditions were an initial denaturation at 95°C for 5 min followed by 35 cycles at 94°C for 1 min (denaturation), 57°C for 1 min (annealing), 72°C for 1 min (extension) and 72°C for 7 min (final extension).

This resulted in a PCR product of 252 bp spanning from position −522 to −774 in the IL-4 promoter region [18]. Ten microliters of the PCR product (252 bp in length) was digested for 16 h at 37°C with Faq I (Fermentas, Vilnius, Lithuania). The promoter −590 C→T polymorphism abolishes the cleavage site for Faq 1 restriction enzyme, resulting in a full length DNA of 252 bp. Heterozygous individuals were identified by the presence of bands of 252, 192 and 60 bp. Digestion products (192 bp and 60 bp) were identified on a 3% Numicropor agarose (Prona, Madrid, Spain) gel.

Statistical analysis

Statistical analysis was done using the SPSS 13.0 for Windows, release 13.01 (SPSS, Chicago, Ill., USA). Distribution of the genotypes for the healthy control and periodontitis groups was checked for Hardy-Weinberg equilibrium. A population is said to be in Hardy-Weinberg equilibrium when all the markers are in Hardy-Weinberg equilibrium p^2(AA), 2pq(Aa), q^2(aa) [31]. The frequencies of the alleles and genotypes in patients and controls were compared by chi-square test and also by the Fisher exact test for small expected counts. Odds ratios and the 95% confidence intervals (95% CI) were calculated. A probability value of $p<0.05$ was considered significant.

Results

The frequency of the *IL-4* gene polymorphisms in a group of 75 patients with CP was compared with that in 73 healthy control subjects. We tested Hardy-Weinberg equilibrium separately for both loci and for cases and controls. Genotype frequencies did not deviate significantly from Hardy-Weinberg equilibrium (HWE; $\chi^2=1.601$, $p=0.449$; $\chi^2=3.57$, $p=0.168$) for polymorphism in intron 2 and promoter, respectively.

Figure 1 is an example of homozygote for the 183 bp polymorphism (line 1) with the intron polymorphism positive (IP+) genotype. When the distribution of IP genotypes is compared between CP patients and healthy controls, no significant difference was observed ($p=0.136$). The positive genotype (183 bp allele) was not associated with CP ($p=0.789$; OR = 1.18, 95% CI: 0.30–4.73). The allele frequencies between CP and control groups were not significantly different ($p=0.185$; OR = 0.71, 95% CI: 0.41–1.22) (Table I).

Figure 2 shows examples of promoter polymorphism. Similar to the IP+ genotype, when the distribution of PP genotypes was compared between CP patients and healthy controls no significant difference was observed ($p=0.775$). The PP+ genotypes (TT) ($p=0.719$; OR = 0.600, 95% CI: 0.138–2.607) and allele frequencies ($p=0.626$; OR = 1.21, 95% CI: 0.672–2.178) were not significantly different between CP and control groups (Table I).

Analysis of composite genotypes showed no tendency for an explanatory contribution of the negative

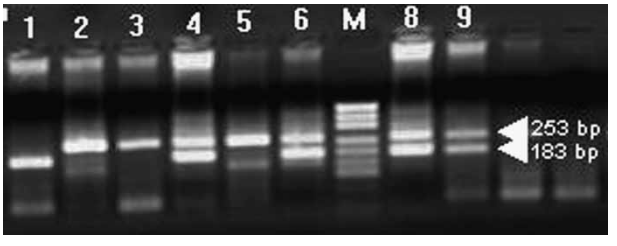

Figure 1. Analysis of the IL-4 intron 2, 70 bp repeat polymorphism. Band 1, homozygous allele 1; bands 2, 3 and 5 homozygous allele 2; and bands 4, 6, 8 and 9 heterozygotes. Lane M. The size marker (pUC19 DNA/MspI) contains the following 13 discrete fragments (in base pairs): 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 34, 26.

Table I. Genotype and allele frequencies for the intron and promoter polymorphisms in the IL-4 gene in CP patients and healthy controls in a Turkish population.

Variable	Patients ($n=75$) n (%)	Controls ($n=73$) n (%)	χ^2	p	OR (95% CI)
IL-4 intron 2 70 bp repeat Genotypes					
1,1 (183 bp)	6 (8)	5 (6.8)		0.136	
1,2 (183/253 bp)	23 (30.7)	34 (46.6)			
2,2 (253 bp)	46 (61.3)	34 (46.6)			
Genotype IP+	6 (8)	5 (6.8)	0.07	0.789	1.18 (0.30–4.73)
Genotype IP−	69 (92)	68 (93.2)			
Allele 1 (polymorph.)	35 (23.3)	44 (30.1)	1.75	0.185	0.71 (0.41–1.22)
Allele 2 (wild type)	115 (76.7)	102 (69.9)			
IL-4 promoter −590 C to T Genotypes:					
TT	5 (6.6)	3 (4)		0.775	
CT	20 (26.7)	19 (26)			
CC	50 (66.7)	51 (70)			
Genotype PP+	5 (6.7)	3 (4.1)		0.719*	0.600 (0.138–2.607)
Genotype PP−	70 (93.3)	70 (95.9)			
Allel T (polmorph.)	30 (20)	25 (17.1)	0.237	0.626	1.21 (0.672–2.178)
Allel C (wild type)	120 (80)	121 (82.9)			
Total of alleles	150	146			
PP+/IP+	2 (2.7)	3 (4.1)		0.679*	0.639 (0.104–3.941)
PP−/IP−	73 (97.3)	70 (95.9)			

*Fisher exact test.

Genotype positive (IP+ and PP+) includes patients homozygous for the polymorphism(s); genotype negative (IP− and PP−) includes patients heterozygous and homozygous negative for the polymorphism(s).

(PP−/IP−) genotype versus the positive (PP+/IP+) genotype ($p=0.679$; OR = 0.639, 95% CI: 0.104–3.941) (Table I).

Discussion

The present investigation was carried out to study the association of *IL-4* gene polymorphisms with severe generalized chronic periodontitis. This is the first study in which polymorphic markers in the intron 2 and promoter of the *IL*-4 gene have been used with regard to the severity of periodontal disease in Turkish Caucasians. In our population, the IP genotype and allele frequencies were not significantly different between CP and control groups; nor were the PP genotype and allele frequencies significantly different between CP and control groups. Furthermore, we found no significant differences in PP+ and IP+ composite genotype frequencies between patients and controls. In contrast, Michel et al. [8], in studying 25 early onset periodontitis patients and age-matched healthy controls, found that 27.8% of the patients carried *IL-4* promoter and intron polymorphism positive, while none of the healthy controls presented the same polymorphism ($p<0.01$). The negative and positive findings for this composite genotype and both chronic and aggressive periodontitis populations from different racial and ethnic backgrounds have been demonstrated [1].

Similar to our findings, Scarel-Caminaga et al. [32] studied the 30 CP and 30 controls and found no significant differences in allele and genotype frequencies of the −590 (C→T) polymorphism between control and CP groups in a Brazilian population. And Pontes et al. [33] found no significant differences in the genotype frequencies of 70 bp repeat and −590 (C→T) polymorphisms between control and periodontitis groups in a Brazilian population of African heritage. In the Korean population, two polymorphisms (−590C→T polymorphism and 70 bp repeat polymorphism) were investigated in 32 chronic periodontitis patients and 150 normal controls. There were no significant

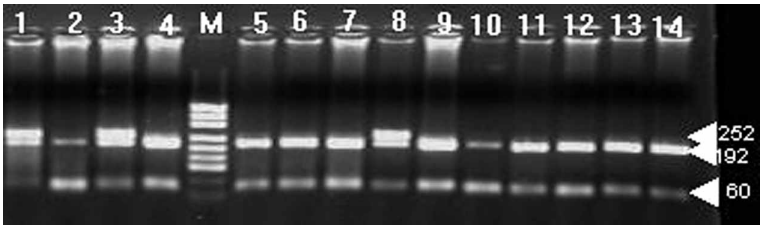

Figure 2. Analysis of the IL-4 −590 (C→T) polymorphism. Bands 1, 3 and 8 heterozygotes (CT); bands 2, 4, 5, 6, 7, 9, 10, 11, 12, 13 and 14 homozygotes (CC). A homozygote TT genotype was not present in this sample set. The size marker is the same as in Figure 2.

differences in the allele, genotype and haplotype distributions of two polymorphisms between normal controls and the chronic periodontitis group [34]. Donati et al. [35] studied 60 patients with severe chronic periodontitis for IL4 receptor gene (IL4RA) polymorphism in Caucasian subjects of a north European origin and found no association with CP.

Gonzales et al. [19], in studying 31 Japanese and 30 Caucasian patients with generalized AgP, plus 30 Japanese and 33 Caucasian healthy controls, found no significant association between IL-4 polymorphisms and the risk of AgP in either population.

In conclusion, we studied the possible association between the IL-4 gene 70 bp repeat and −590 C to T polymorphisms and severe generalized CP in a Turkish population. Our findings indicate that neither genotype nor allele frequencies differ significantly between CP patient and control groups. These findings suggest that IL-4 may not be a candidate genetic risk factor for periodontal disease in the studied population. The conflicting results reported in the literature may stem from ethnicity, small sample size or differing environmental effects on CP.

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