Interleukin-6 promoter polymorphism (-174 G/C) in Indian patients with chronic periodontitis

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(Received 19 January and accepted 21 June 2010)

Abstract: Recent studies have focused on genetic polymorphism of the interleukin-6 (IL-6) gene, which has led to a better understanding of the intricate interactions between host response, microorganisms, and genetics. Genotype prevalence appears to vary by the race and ethnicity of the population studied. We used a polymerase chain reaction technique to determine the prevalence of single nucleotide polymorphism in IL-6 at position -174 G>C in a population of 30 South Indians. Blood samples were collected from 15 chronic periodontitis patients and 15 healthy controls. The results showed that the G/G genotype was significantly more frequent in the chronic periodontitis group and that the C/C genotype was significantly more frequent in the control group (P = 0.0069 for both). The G allele was more frequent in chronic periodontitis patients (76.67%), whereas the C allele was more frequent in the control group (73.33%). Among chronic periodontitis patients, the odds ratio for having the G allele, as compared with the controls, was 9.04. In this population, the presence of the G/G genotype of IL-6 (-174) might increase susceptibility to chronic periodontitis, whereas the C/C genotype may have a protective effect. (J Oral Sci 52, 431-437, 2010)

Keywords: interleukin-6; single nucleotide polymorphism; genotype; chronic periodontitis.

Introduction

There has been considerable new evidence regarding the pleiotropic role of interleukin-6 (IL-6) in host defense (1,2). IL-6 has diverse functions, including differentiation and activation of macrophages and T cells, and growth and terminal differentiation of B cells. This cytokine has been implicated in the pathogenesis of many inflammatory diseases, such as psoriasis (3) and rheumatoid arthritis (4). It is a major mediator of the host response to injury and infection: high levels of IL-6 have been observed in patients with infections, trauma, chronic inflammatory diseases, and neoplasia (5).

IL-6 is produced by activated macrophages, lymphocytes, and adipose tissue (6,7). In addition, it has been shown that, by controlling the level of pro-inflammatory but not anti-inflammatory cytokines, endogenous IL-6 plays a crucial anti-inflammatory role in both local and systemic acute inflammatory responses (8).

Gingivitis and periodontitis are infectious inflammatory diseases of the periodontium initiated by microorganisms...
present in susceptible individuals. Increased levels of interleukin-6 have been found in gingival crevicular fluid in patients with periodontitis (9). It has also been shown that fibroblasts from patients with periodontal lesions constitutively produced greater amounts of IL-6 than did those from healthy controls (10).

IL-6 is considered a useful indicator of periodontal disease (11,12), as levels of this cytokine are higher in sites with gingivitis than in healthy sites (13-15). In addition, higher expression of IL-6 mRNA was reported in diseased tissues than in healthy tissues in patients with periodontitis (16).

A number of studies have focused on the role of interleukin-6 genetic polymorphism in inflammatory systemic diseases and conditions (17-19). There is strong evidence that genetic, as well as environmental factors affect the age of onset, severity, and risk of developing periodontitis (20-22). Studies have found an association between the polymorphism in IL-6 at position -174 G>C and periodontitis in different populations, including white Brazilians (23), Southern Germans (24), Northern Europeans (25), and white Europeans (26). The findings of these studies are inconsistent, however, as some have shown a positive association between this polymorphism and the severity of periodontitis, while others have not. This variation may be due to ethnic differences in the studied populations. Therefore, more research on races from different parts of the world is essential to understand the association of IL-6 with periodontitis.

Using a polymerase chain reaction (PCR) technique, we compared the prevalence of the single nucleotide polymorphism (-174 G>C) in patients with chronic periodontitis and in subjects with healthy periodontium. To our knowledge, this is the first such study conducted in a South Indian population.

**Materials and Methods**

The study population was selected randomly from patients attending the outpatient section of the Department of Periodontics, PMNM Dental College and Hospital, Bagalkot, India from March to September of 2008. Venous blood samples were collected from 15 randomly chosen subjects with healthy periodontium (9 men and 6 women, age range 32-41 years, mean age 36.73 ± 2.74 years) and 15 with chronic periodontitis (9 men and 6 women, age range 33-49 years, mean age 39.20 ± 4.30 years).

Chronic periodontitis was diagnosed using the periodontal disease classification system of the American Academy of Periodontology (1999). Chronic periodontitis patients were defined as those with periodontal pockets greater than 5 mm and clinical attachment loss greater than 3 mm in more than 20 teeth, with moderate to severe bone loss. All patients were systemically healthy and had not received periodontal treatment or antibiotics for at least 6 months before the clinical examination and sampling. Other exclusion criteria were diseases of the oral hard or soft tissues, except caries; chronic use of anti-inflammatory drugs; history of diabetes, hepatitis, or human immunodeficiency virus infection; history of any disease known to severely compromise immune function; current pregnancy or lactation; and history of smoking. Periodontal evaluation included the oral hygiene index simplified, gingival index, plaque index, probing pocket depth, and clinical attachment loss.

Ethical clearance for the study was obtained from the Ethical Review Board of the PMNM Dental College and Hospital, Bagalkot, India, and the subjects who satisfied the inclusion criteria of the study were selected. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki, and all procedures were carried out with the understanding and written consent of the subjects.

**Collection of samples**

Venous blood samples (2 ml) were collected from the antecubital fossa from all subjects, after obtaining their consent to participate in the study. The whole blood samples thus collected were transferred to aliquots containing EDTA and sent to a laboratory for PCR analysis of single nucleotide polymorphism of IL-6. The collection of blood samples was approved by the ethical review board.

**DNA extraction**

Approximately 100 µl of the blood sample was mixed with Tris-EDTA (TE) buffer incubated for 5 min and then washed repeatedly with the same buffer to obtain cell sediment. Then, 400 µl of lysis buffer I (4 M guanidinium thiocyanate, 0.5% N-lauroylsarcosine, 1 mM dithiothreitol, 25 mM sodium citrate, and 40 µg of glycogen/tube) was added, incubated for 5 min, and centrifuged again. The supernatant was discarded and lysis buffer II (Tris-HCL, Nonidet-P40, Tween 20, and freshly prepared proteinase K 100 µg/ml) was added to the sediment. The tubes were kept at 65°C in a water bath for 2 h, followed by boiling for 10 min. The tubes were cold-stored at -20°C until processed by the PCR technique. DNA amplification was done using hot-start PCR.

**Primers**

The PCR primers were synthesized with an Applied Biosystems oligonucleotide synthesizer (Lab India
Instruments Pvt. Ltd., Haryana, India). Primer pair 5’ TGACTTCAGCTTTACTCTTTGT 3’ and 5’ CTGATTGGAACCTTAT TAAG 3’ was chosen for the study.

DNA amplification

The amplification was performed with a Perkin-Elmer Gene Amp PCR system 9600 (Lab Centraal BV, Haarlem, Netherlands). Forty amplification cycles of 30 s at 94°C, 40 s at 60°C, and 50 s at 72°C were carried out with a final volume of 50 µl containing 5 µl of 10x reaction buffer, 0.2 mM dNTP, 10 pmol of each of 12 primers, and 2.5 U of cloned Pfu DNA polymerase (Bangalore Genei, Bangalore, India). Five microliters of appropriate DNA samples (positive controls) were added to the reaction mixture. After the final cycle, the samples were incubated for 15 min at 78°C to complete the extension of primers (27).

Confirmation of PCR products with restriction enzyme

To the 10 µl of PCR reaction mixture, 16 µl of nuclease-free water, 2 µl of 10x buffer tango, and 1-2 µl of SfaNI enzyme (New England Biolabs, Lab Mate Asia Pvt. Ltd., Madras, India) was added. Then the mixture was gently mixed and spun down for a few seconds. The mixture was then incubated at 37°C for 1-6 h and thermal inactivation was performed. SfaNI was inactivated by incubation at 65°C for 20 min. SfaNI is an enzyme that cuts the DNA sequences at GCATC (5/9) and is ideal for cleaving amplified products of IL-6 into distinct fragments for identification of gene polymorphism with the set of primers used in the study. Ten microliters of each amplified product was analyzed by agarose gel electrophoresis on 2% agarose (NUSIEVE 3:1; FMC, Rockland, ME, USA) containing 1 µg of ethidium bromide/ml in 1x TBE buffer, and was visualized in an ultraviolet transilluminator (Fotodyne, Hartland, WI, USA).

Statistical analysis

The chi-square test was used to analyze the allele ratio and genotype distribution of periodontitis patients and healthy controls. Odds ratios were used to measure the strength of the associations between risk factors and outcome. A P value of <0.05 was considered significant. STATA version 9.2 (STATA Corp LP, College Station, TX, USA) was used for the analysis of the data; Microsoft Word was used to generate the tables.

Results

This was a case-control study of 30 subjects divided into 2 groups. One group included 15 randomly chosen subjects with healthy periodontium the other group consisted of 15 chronic periodontitis patients in the age range of 33-49 years (mean age of 39.20 ± 4.30 years). The numbers of males (n = 9) and females (n = 6) in both groups were equal. The prevalence of the single nucleotide polymorphism of IL-6 in the promoter region -174 (G>C) was compared.

The genotypes in subjects with healthy periodontium were as follows (Table 1): G/G in 2, G/C in 4, and C/C in 9 subjects. The genotypes in the chronic periodontitis patients were as follows (Table 1): G/G in 10, G/C in 3, and C/C in 2 patients. There was a significant difference in the distribution of the IL 6 -174 alleles between healthy controls and periodontitis patients (P = 0.0069).

A significant (P < 0.01) difference between groups in the frequencies of the G and C alleles was noted (Table 2). The G allele was found in 23 (76.67%) of 30 alleles in the chronic periodontitis subjects (n = 15), and in 8

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>Percentage</th>
<th>Chronic Periodontitis</th>
<th>Percentage</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>2</td>
<td>13.33</td>
<td>10</td>
<td>66.67</td>
<td>12</td>
</tr>
<tr>
<td>G/C</td>
<td>4</td>
<td>26.67</td>
<td>3</td>
<td>20.00</td>
<td>7</td>
</tr>
<tr>
<td>C/C</td>
<td>9</td>
<td>60.00</td>
<td>2</td>
<td>13.33</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>100.00</td>
<td>15</td>
<td>100.00</td>
<td>30</td>
</tr>
</tbody>
</table>

Chi-square statistic with 2 degrees of freedom = 9.9310; P = 0.0069

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control</th>
<th>Percentage</th>
<th>Chronic Periodontitis</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>8</td>
<td>26.67</td>
<td>23</td>
<td>76.67</td>
</tr>
<tr>
<td>C</td>
<td>22</td>
<td>73.33</td>
<td>7</td>
<td>23.33</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>100.00</td>
<td>30</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Odds ratio = 9.04, P < 0.01
(26.67%) of 30 alleles in the control group. The frequency of the C allele was greater in the control group: 22 (73.33%) of 30 alleles in chronic periodontitis patients. For the G allele versus the C allele, the odds ratio (chronic periodontitis patients versus controls) was 9.04.

The distribution of genotypes by sex is listed in Table 3. Among the 18 men, 6 had the G/G genotype, 6 had G/C, and 6 had C/C. Among the 12 women, 6 had the G/G genotype, 1 had G/C, and 5 had C/C. The difference between sexes was not statistically significant.

### Discussion

To the best of our knowledge, this is the first study conducted in a South Indian population of the prevalence of single nucleotide polymorphism of IL-6 (-174 G>C) in chronic periodontitis patients. Genetic polymorphism was assessed using a PCR technique.

The overall genotype distribution was significantly different between groups. The results showed that the G/G genotype was more frequent in the periodontitis group and that the C/C genotype was more frequent in controls (\(P = 0.0069\)). Patients with periodontitis had a significantly higher frequency of -174 G/G homozygosity (66.67%) than did controls (13.33%), suggesting that the -174 G/G genotype plays a role in the progression of chronic periodontitis. Similar results were found in a study by Trevilatto et al. (23), who reported that the G/G genotype was significantly associated with susceptibility to chronic periodontal disease. They suggested that the G allele plays a role in the pathogenesis and development of periodontal disease. The main finding of a study by Tervonen et al. (28) was that in patients with periodontitis, the T allele of CD14 -260 and the GG genotype of IL-6 -174 were independently associated with advanced periodontal disease, which is in agreement with the findings of the present study. These findings also support the hypothesis that a positive genotype predisposes individuals to periodontitis. Recently, Costa et al. (29) suggested that the IL-6 gene polymorphism may be associated with chronic periodontitis in elderly patients. In another study, Moreira et al. (30) provided support for the hypothesis that IL-6 gene polymorphisms and haplotypes are moderately associated with periodontitis.

In our study, the C/C genotype was more prevalent in healthy subjects (60%) than in chronic periodontitis patients (13.33%), indicating that this genotype might reduce susceptibility to chronic periodontitis. A study conducted by Fishman et al. (18) showed that patients with systemic-onset juvenile arthritis had a lower frequency of the C/C genotype, indicating that this genotype protects against the development of this condition. However, in a study conducted by Babel et al. (31), the -174 IL-6 CC genotype, which codes for IL-6 low phenotypic production, was more frequent in periodontitis patients than in controls (\(P = 0.0283\)). Moreover, D’Aiuto et al. (32) found that high serum levels of IL-6 in periodontitis patients were associated with carriage of the C allele.

In the present study, the prevalence of the G/C genotype was marginally higher in controls (26.67%) than in chronic periodontitis patients (20.00%). Trevilatto et al. (23) reported similar results, namely, that the frequency of the G/C genotype decreased as the severity of periodontal disease increased: 58.3% with this genotype were healthy, 50% had moderately severe disease, and only 12.5% had severe disease. Allele C might reduce IL-6 production, which explains its protective function. However, the -174 G/C polymorphism was reported to influence the development of septicemia in preterm infants (33), and Holla et al. (34) found no association between this polymorphism and chronic periodontitis in white Czechs.

In the present study, significant results were observed in the distribution of G and C alleles (odds ratio for chronic periodontitis patients versus controls, 9.04; \(P < 0.01\)). The G allele was present in 76.67% of the alleles of chronic periodontitis patients and in only 26.67% of those of controls. Moreover, while the C allele was present in 73.3% of the alleles of controls, it was present in only 23.3% of the alleles of chronic periodontitis patients. Our findings indicate that subjects with the G allele were at 9 times the risk for chronic periodontitis as those with the C allele. In other studies, the -174 G allele was more frequent in periodontitis patients than in healthy controls (23,35). Moreira et al. (36) suggested that the G genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>6 (33.33%)</td>
<td>6 (50%)</td>
<td>12</td>
</tr>
<tr>
<td>G/C</td>
<td>6 (33.33%)</td>
<td>1 (8.33%)</td>
<td>7</td>
</tr>
<tr>
<td>C/C</td>
<td>6 (33.33%)</td>
<td>5 (41.67%)</td>
<td>11</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>18</strong></td>
<td><strong>12</strong></td>
<td><strong>30</strong></td>
</tr>
</tbody>
</table>
reduced the severity of periodontitis in a Brazilian population.

The C allele was associated with significantly lower levels of plasma IL-6 in a study by Fishman et al. (18), who maintained that a single nucleotide change from G to C at position -174 results in suppression of IL-6 transcription in response to lipopolysaccharide. The fact that the 115-bp fragment does not elicit high basal expression suggests that sequences between -225 and -164 exert a negative influence on this promoter and thus a negative regulatory effect on gene expression (37).

The results of studies associating genetic polymorphism of the IL-6 gene and periodontitis are inconsistent. A positive association of the single nucleotide polymorphism of IL-6 (-174G>C) with the severity of periodontitis was found in some studies, while other studies found no such association (23-26). If the prevalence of this polymorphism varies in different populations, the association with susceptibility to periodontitis might also vary. This would lead to discrepancies in genetic polymorphism studies conducted on periodontitis patients of different ethnicities and diverse genetic backgrounds. Apart from racial differences, the differences might occur due to a number of confounding factors, including clinical diagnoses, environmental variables, biologic plausibility, penetrance, and logic of association studies (38).

In conclusion, the results of the present study of South Indian patients show that the G/G genotype is more frequent in chronic periodontitis patients than in healthy controls, and that the reverse is true for the C/C genotype. This suggests that the G/G (-174) genotype of IL-6 cytokine increases susceptibility to chronic periodontitis and that the C/C genotype protects against the development and progression of chronic periodontitis. The inconsistent results yielded by studies of IL-6 polymorphisms emphasize the need for more research on different populations with diverse genetic backgrounds. If such research is conducted, the association between chronic periodontitis and the single nucleotide polymorphism in IL-6 at position -174 G>C can be characterized globally.

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