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Analysis of the MMP-9 (C-1562 T) and TIMP-2 (G-418C) gene promoter polymorphisms in patients with chronic periodontitis

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Abstract

Background: Matrix metalloproteinases (MMP)-9 is an important member of the matrix metalloproteinase family. A functional polymorphism has been described in the promoter region of the human *MMP-9* gene. A C-to-T base exchange at − 1562 creates two different alleles, and the C/T and T/T genotypes promote high activity of the *MMP-9* gene promoter, increasing the risk for inflammatory diseases. The metalloproteinase-2 tissue inhibitor (TIMP-2) regulates the activity of MMPs in the extracellular matrix, and a polymorphism at the − 418 position of the *TIMP-2* gene promoter has been found in a Sp-1 binding site. In this study we have investigated the association between the above-mentioned polymorphisms and chronic periodontitis severity.

Methods: Genomic DNA from oral mucosa of 100 subjects was amplified by polymerase chain reaction and analysed by restriction endonuclease digestion. The significance of the differences in observed frequencies of polymorphisms in moderate and severe disease and healthy groups was assessed by χ^2 test ($p < 0.05$).

Results: No association was observed between the polymorphism in the promoter region of *MMP-9* ($p = 0.6693$) and chronic periodontitis. The analysis of *TIMP-2* showed that the G/G genotype was found at a frequency of 99%.

Conclusion: The results show that the polymorphism in the promoter region of *MMP-9* gene is not associated with chronic periodontitis. The high frequency of GG genotype in the *TIMP-2* gene promoter in the population studied did not allow any conclusion regarding its effect on chronic periodontitis.

Key words: chronic periodontitis; gelatinase; MMP-9; polymorphism; TIMP-2

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Matrix metalloproteinases (MMPs) constitute an important family of zinc-dependent endopeptidases, which are able to degrade most, if not all, components of extracellular matrix (ECM) (Birkedal-Hansen 1993). These enzymes have been classified into groups in accordance to substrate affinity and structural design. At least 20 MMPs have been described and the matrix metalloproteinase-9 (MMP-9), also known as gelatinase B, is highly expressed during periodontitis (Ingman et al. 1996, Seguier et al. 2001). This enzyme is expressed by polymorphonuclear (PMN)-leukocytes, macrophages, keratinocytes and endothelial cells (Birkedal-Hansen 1993), being active against connective tissue proteins, such as types IV, V and XI collagen, proteoglycans and elastin (Vu & Werb 1998). MMP-9 is secreted by cells as zymogen and is activated by serine proteinases or even by other MMPs. MMP-9 activity is under strict control at several levels. At the transcription level, the expression of MMP-9 is controlled in response to regulatory molecules, such as tumor necrosis factor-α, interleukin (IL)-1, platelet-derived growth factor and epidermal growth factor (Fabunmi et al. 1996, Kondapaka et al. 1997, Opdenakker et al. 2001). The activity of metalloproteinases on the ECM is regulated by specific MMPs inhibitors, known as tissue metalloproteinases inhibitors (TIMPs). The TIMP family consists of four members: TIMP-1, -2, -3 and -4. These molecules inhibit the proteolytic activity of activated MMPs by forming a

1:1 stochiometric inhibitory complex with the enzyme. Disturbances in the MMP–TIMP interactions have been implicated in the etiology of some diseases, such as arthritis, pulmonary emphysema, atherosclerosis and periodontitis, in which the loss of ECM is a major feature (Hammani et al. 1996).

A number of studies have focused on the association of genetic polymorphisms and susceptibility of or severity to periodontal disease (Kornman et al. 1997, Diehl et al. 1999, Engerbretson et al. 1999, Michalowicz et al. 2000, Socransky et al. 2000, Scarel-Caminaga et al. 2002, 2003, de Souza et al. 2003a, b, Trevilatto et al. 2003), encouraging the search for genetic markers for periodontitis. The *MMP-9* gene presents a functional C-to-T single nucleotide polymorphism (SNP) at position −1562, which affects transcription. The CC genotype has been shown to decrease transcriptional activity (Zhang et al. 1999). This *MMP-9* polymorphism has been associated with high risk for vascular diseases, such as coronary atherosclerosis (Zhang et al. 1999, Pöllänen et al. 2001, Wang et al. 2001).

A polymorphism in the *TIMP-2* gene promoter at position −418 has been described, and corresponds to a G to C substitution in the consensus sequence for the Sp1 binding site (Hirano et al. 2001). Polymorphisms in the promoter region of *TIMP-2* gene have been associated with chronic obstructive pulmonary disease (Hirano et al. 2001). This study intended to correlate polymorphisms in the promoter region of the *MMP-9* and *TIMP-2* genes to chronic periodontal disease.

Material and methods

Subject selection

A convenience sample of 100 unrelated, non-smoking subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba – UNICAMP. The patients are from the Southeastern region of Brazil. The age, gender and ethnic group of the subject population are presented in Table 1. All subjects were in good general health and had at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for premedication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute narcotizing ulcerative gingivitis or current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth (PD), assessment of attachment loss (CAL), tooth mobility and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at six points around each tooth. Subjects were included in clinical categories according to PD severity:

Table 1. Age, gender and ethnic group of the subject population ($n = 100$)

	Healthy ($n = 38$)	Moderate ($n = 27$)	Severe ($n = 35$)
Age (years) Mean (± SD)	43.2 (± 14.0)	36.9 (± 11.2)	43.6 (± 14.4)
Gender (%)			
Female	68.2	80.6	84.2
Male	31.8	19.4	15.8
Ethnic group (%)			
Caucasoid	81.5	77.4	68.4
Afro-American	7.9	16.1	13.2
Mulatto	8.0	6.5	18.4
Japanese	2.6	0.0	0.0

(1) *Healthy group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of CAL and no sites with PD >3 mm ($n = 38$)
(2) *Moderate periodontitis*: Patients with at least three teeth exhibiting sites with CAL ⩾3 mm and <7 mm, in at least two different quadrants ($n = 27$)
(3) *Severe periodontitis*: Patients with at least three teeth exhibiting sites with CAL ⩾7 mm, in at least two different quadrants ($n = 35$)

Sampling

The sampling of buccal epithelial cells was performed as described by Trevilatto & Line (2000). Briefly, individuals undertook a mouthwash after 1 min., containing 5 ml 3% glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shacked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 5000 g for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 µl of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM ethylenediamine tetraaceticacid (EDTA), 0.5% sodium dodecyl sulphate (SDS)]. The samples were then frozen at −20°C until used for DNA extraction.

DNA Extraction

After defrosted, samples were incubated overnight (ON) with 100 ng/ml proteinase K (Sigma Chemical Co., St Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 µl TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

PCR

The sequence from −1809 to −1374 in the *MMP-9* gene promoter was PCR amplified with primers 5′-GCCTGGCA CATAGTAGGCCC-3′ (forward) and 5′-CTTCCTAGCCAGCCGGCATC -3′ (reverse). PCR was carried out in a total volume of 50 µl, containing 500 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 µM of each primer, 200 µM each dATP, dCTP, dGTP and dTTP, and 2.5 U *Taq* DNA polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The solution was incubated for 3 min. at 95°C, followed by 35 cycles of 1 min. at 95°C, 45 s at 65°C and 45 s at 72°C, with a final extension of 72°C for 7 min.

A fragment of 176 bp of the *TIMP-2* gene promoter was PCR amplified with primers 5′-GGATCCTGTCAGTTTCT-CAA-3′ (forward); 5′-TTTCCCCTTAG CTCGACTCT-3′ (reverse). PCR was carried out in a total volume of 50 µl, containing 500 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 µM of each primer, 200 µM each dATP, dCTP, dGTP and dTTP, and 4 U Taq DNA polymerase (Amersham Pharmacia Biotech AB). The solution was incubated for 3 min. at 95°C, followed by 35 cycles of 1 min. at 95°C, 1 min. at 55°C and 1 min. at 72°C, with a final extension of 72°C for 7 min.

Restriction endonuclease digestion (RFLP)

A 2 µl aliquot of *MMP-9* PCR products was mixed with a 8 µl solution containing 1 µl 10 × NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.3 µl *Pae*I (20,000 U/ml) (New England Biolabs, Inc., Beverly, MA, USA) and 6.7 µl sterile deionized H_2O. The solution was incubated at 37°C ON. For analysis of *TIMP-2* polymorphism a 3 µl aliquot of TIMP-2 PCR products was mixed with a 17 µl solution containing 2 µl 10 × NE Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.5 µl *Hga*I (2000 U/ml) (New England Biolabs, Inc.) and 14.5 µl sterile deionized H_2O. The solution was incubated at 37°C ON.

Gel electrophoresis

The total amount aliquot of the digest was mixed with 3 µl of loading buffer and electrophoresed on a 10% vertical non-denaturing polyacrylamide gel at 20 mA. The gel was silver stained by DNA Silver Staining Kit (Amersham Pharmacia Biotech AB).

Statistical analysis

The significance of the differences in observed frequencies of the polymorphisms among the groups (healthy, moderate and severe disease) was assessed by the χ^2 test, and a $p < 0.05$ was considered statistically significant.

Results

Table 1 shows the age, gender and ethnic group of the subject population.

Table 2. Distribution of the *MMP-9*alleles in healthy, moderate and severe groups with chronic periodontitis

Allele	Healthy (*n*, %)	Moderate (*n*, %)	Severe (*n*, %)	*p* Value
C	61 (80.3)	44 (81.5)	60 (85.7)	0.6693
T	15 (19.7)	10 (18.5)	10 (14.3)	

MMP, matrix metalloproteinase.

Table 3. Distribution of the *MMP-9* genotypes in the healthy group, and in groups with moderate and severe chronic periodontitis

Genotype	Healthy (*n*, %)	Moderate (*n*, %)	Severe (*n*, %)	*p* Value
C/C	24 (63.2)	17 (63.0)	25 (71.4)	0.6935
C/T	13 (34.2)	10 (37.0)	10 (28.6)	
T/T	01 (02.6)	0.0 (0.0)	0.0 (0.0)	

MMP, matrix metalloproteinase.

The results show that there is no association between the allele frequencies for the polymorphism in the *MMP-9* gene promoter and chronic periodontitis ($p = 0.6693$). The same was found for the different genotypes ($p = 0.6935$). The TT genotype, which is believed to increase *MMP-9* transcription, was not observed for the moderate and severe periodontitis groups. This genotype was observed in only one individual in the healthy group. The C allele was observed at a frequency of 80.3%, 81.5% and 85.7%, and the T allele was found at frequency of 19.7%, 18.5% and 14.3%, in the healthy, moderate and severe periodontitis groups, respectively (Table 2). The CT genotype was observed at frequencies of 34.2%, 37.0% and 28.6% in the healthy, moderate and severe periodontitis groups, respectively (Table 3). When only Caucasian individuals were analysed, similar results were obtained. The *TIMP-2* polymorphism analysis showed that only one G allele was observed in the population. It was present in a heterozygous individual of Japanese origin.

Discussion

Gene polymorphisms are a mechanism by which individuals may exhibit variations within the range of what is considered biologically normal. Single nucleotide polymorphisms (SNPs) occur at a high frequency in the human genome and can affect the function of genes. Various SNPs have been found in the promoter region of several MMPs, and they have been associated with different diseases (Ye 2000). Studies have demonstrated an association between the presence of the −1562 SNP in the MMP-9 gene promoter and some inflammatory diseases (Zhang et al. 1999, Pöllänen et al. 2001, Wang et al. 2001). A C-to-T exchange at position −1562 alters the binding of a nuclear protein to this region, leading to increased transcriptional activity in macrophages (Zhang et al. 1999). MMP-9 is the most complex MMP family member in terms of protein structure and regulation of its activity (Opdenakker et al. 2001). In contrast to other gelatinases (such as MMP-2 or gelatinase A), MMP-9 is produced by a limited number of cells and its production is not constitutive (Murphy & Knäuper 1997, Vu & Werb 1998, Opdenakker et al. 2001). The expression of MMP-9 is primarily controlled at the transcriptional level, where the promoter of the gene responds to stimuli of various cytokines and growth factors (Huhtala et al. 1991, Kondapaka et al. 1997).

Periodontitis patients have significantly higher levels of MMP-9 in their oral rinses and crevicular fluid than healthy subjects (Makela et al. 1994). Neutrophils are the first-line defense leukocytes in periodontal disease (Opdenakker et al. 2001). These cells store large amounts of MMP-9 in intracellular granules and the rate of degranulation has been pointed as the major factor that determines quiescence and acute phases of periodontal disease (Sodek & Overall 1992). The levels of MMP-9 in gingival crevicular fluid have been used as a marker for periodontitis stage (Ingman et al. 1996). Moreover, potent MMP-9 inductors are found in the gingival crevicular fluid of periodontitis patients, such as lipopolysac-

charide (LPS), a microbial product derived of the cellular-membrane rupture (Opdenakker et al. 2001, Vu & Werb 1998), and IL-8 that is considered to be the major human neutrophil chemoattractant. IL-8 induces the release of MMP-9 from cells, and this enzyme in turn cleaves IL-8 creating a ten-fold more active fragment of the chemokine (Opdenakker et al. 2001). Moreover, MMP-9 is believed to modulate bone matrix degradation. Osteoclasts normally produce MMP-9 (Okada et al. 1995). mRNA levels of MMP-3, MMP-13 and MMP-9 in osteoclasts are increased when cells are stimulated by bone resorptive factors (Uchida et al. 2000).

Although MMP-9 plays an important role in ECM destruction, we could not find a relationship between -1562 polymorphism and the different levels of chronic periodontal disease. Another study has also found a negative correlation between this polymorphism and multiple sclerosis (Nelissen et al. 2000).

An imbalance between MMPs and TIMPs synthesis can promote destruction of the ECM components (Ryan et al. 1996). The single base G/C substitution at position -418 of the *TIMP-2* gene promoter is located in a Sp1 consensus sequence (Faisst & Meyer 1992). A study of the *TIMP-2* promoter activity demonstrated that a 519 bp segment upstream the major transcription initiation site contains active promoter elements (De Clerck et al. 1994). It has been suggested that the -418-nucleotide substitution in the consensus sequence for Sp1 results in downregulation of the transcription activity of the *TIMP-2* gene (Hirano et al. 2001).

The high frequency of GG genotype of the *TIMP-2* gene promoter in the population studied did not allow any conclusion regarding the effect of the studied polymorphism on chronic periodontitis. However, this polymorphism was associated with the development of chronic obstructive pulmonary disease in a Japanese population (Hirano et al. 2001). That Japanese population showed heterozygous genotypes at frequency of 29.68% (Hirano et al. 2001), suggesting that this variant could be common in Oriental subjects. Interestingly, in our study the only subject who had a C allele was a Japanese descendent. Among Caucasian subjects of the Southeastern region of Brazil the frequency of this polymorphism in the *TIMP-2* gene appears to be very low.

The finding of specific alleles that modify the expression of MMPs and their tissue inhibitors (TIMPs) could provide important insights on the pathogenesis of diseases, indicating individuals of high risk. Furthermore, the diagnosis of alleles that impute disease risk could indicate the use of alternative therapy. MMPs inhibitors are a promise of treatment for diseases in which MMPs activity is up regulated (Nagase & Brew 2002, Rudek et al. 2002). They will certainly be applicable in periodontal disease treatment in the near future.

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