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MMP-2, MMP-9 and TIMP-2 gene polymorphisms in Chinese patients with generalized aggressive periodontitis

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Abstract

Background: Aggressive periodontitis (AgP) has a genetic basis. It has been reported that the functional gene polymorphisms of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase-2 (TIMP-2) alter their expressions in transcriptional level and they are involved in the tissue destruction of periodontitis. The study was carried out to analyse the association of functional polymorphisms in MMP-2, MMP-9 and TIMP-2 with generalized AgP (G-AgP) in a Chinese population.

Material and Methods: The study population consisted of 79 Chinese patients with G-AgP and 128 healthy controls. DNA was obtained from oral mucosa swab samples. MMP-2 genotypes were determined by PCR-based denaturing high-performance liquid chromatography analysis while MMP-9 and TIMP-2 genotypes were identified by a PCR-based restriction fragment length polymorphism. χ^2 test after Yates' correction was used to investigate the possible association of the genotypes with the G-AgP.

Results: Although gene polymorphisms for MMP-2 and MMP-9 did not show any association with the G-AgP, the analysis of the TIMP-2 -418G to C gene polymorphism revealed significant differences between the patients and controls. Compared with controls, a significant increasing trend of TIMP-2 -418C carrier in the G-AgP patients occurred ($p = 0.013$).

Conclusion: It is suggested that the TIMP2 -418G to C gene polymorphism is associated with G-AgP in the Chinese subjects.

Key words: aggressive periodontitis; matrix metalloproteinase; polymorphism; tissue inhibitor of metalloproteinase

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Periodontal diseases, involving microbial challenge and host responses, are one group of the most common disorders. Although bacteria are an initial factor for human periodontitis, their impact may be modified by an individual's predisposition. Aggressive periodontitis (AgP), as a subgroup of periodontitis, is characterized by rapid degradation and destruction of periodontal supporting tissue in otherwise clinically healthy juveniles or early adults (Armitage 1999, Tonetti & Mombelli 1999). It has been suggested that there is a genetic basis and a predisposition for individuals to suffer from AgP (Beaty et al. 1987, Hodge & Michalowicz 2001, Nibali et al. 2006, Rabello et al. 2006).

Studies to date have focused on the relationship between genetic variation in the candidate genes of the immune and pro-inflammatory systems and chronic periodontitis (CP) or AgP (Kornman et al. 1997, Fu et al. 2002, Machulla et al. 2002, Soga et al. 2003, Li et al. 2004, Brett et al. 2005, Atilla et al. 2006). The pro-inflammatory cytokines stimulate cells of the host to produce a number of matrix metalloproteinases (MMPs), which are eventually responsible for degradation of periodontal connective tissues in the pathogenesis of periodontitis (Birkedal-Hansen 1993). MMPs perform multiple roles in the host response

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to the progression of infection, facilitating leucocyte recruitment, cytokine and chemokine processing and matrix remodeling (Ryan & Golub 2000). MMP-2 and MMP-9 (named as gelatinase A and gelatinase B), mainly cleaving type IV collagen, are also believed to play important roles in tissue destruction in periodontitis (Ingman et al. 1994, Makela et al. 1994, Korostoff et al. 2000, Ejeil et al. 2003). Elevated MMP-2 and MMP-9 levels of tissue or gingival crevicular fluid (GCF) have been observed in inflammatory sites in periodontitis (Ejeil et al. 2003, Pozo et al. 2005).

It has been reported that the functional polymorphisms existed in MMPs and TIMPs genes. A cytosine (C) to thymine (T) substitution at position -1306 bp relative to the transcriptional start site of MMP-2 gene changes *cis*-regulatory elements by disrupting a Sp1-type promoter site (CCACC box) (Price et al. 2001). The position -1562 bp in the MMP-9 promoter region also has a C to T substitution, which has an allele-specific effect on MMP-9 transcription (Zhang et al. 1999). Overproduction of MMPs resulting from these polymorphisms may influence the manifestation and development of periodontal diseases.

Tissue inhibitor of metalloproteinase-2 (TIMP-2) is particularly interesting because of its dual functions in terms of regulating MMP-2 activity (Reynolds 1996, Robert & Hideaki 2003). A guanine (G) to C transition located at -418 bp has also been identified in the consensus sequence for the Sp1-binding site in the promoter region of TIMP-2 (Hirano et al. 2001). It is reasonable to postulate that the polymorphism may down-regulate TIMP-2 expression and consequently cause an imbalance between the activities of TIMP-2 and MMP-2, which are believed to have a significant impact on periodontitis development and progression. However, the relationship between their polymorphisms and AgP susceptibility is as yet unclear. Thus, we hypothesized that the polymorphisms of MMP-2, MMP-9 and TIMP-2 are genetic risk factors of AgP.

As the investigation was conducted among the Chinese population, we were particularly concerned about whether genetic variations of MMP-2, MMP-9 and TIMP-2 are gene biomarkers for susceptibility to G-AgP in Chinese subjects.

Material and Methods

Subject selection

A total of 207 unrelated Chinese individuals were selected for the study population, consisting of 79 G-AgP patients and 128 periodontally healthy volunteers serving as the control group. All participants were ethnically homogeneous Han Nationality. G-AgP patients were enrolled from the Department of Periodontology, School of Stomatology, Fourth Military Medical University, Xi'an city, China. The diagnostic criteria for G-AgP were based on the 1999 International Classification of the Periodontal Disease and Conditions (Armitage 1999). Diagnoses were confirmed by periodontal examination, consisting of the probing pocket depth, clinical attachment loss (CAL) at six sites per teeth by using a constant-force Florida Electronic Probe® (Florida Probe, Gainsville, FL, USA) and full-mouth periapical radiographs. The additional clinical criteria reported previously (Tonetti & Mombelli 1999) were also used:

(1) patients should be aged 35 years or less at diagnosis and be systemically healthy;
(2) at least six teeth, at least three of which are not first molars or incisors, should have a probing depth $\geqslant 5$ mm and CAL $\geqslant 4$ mm; in the G-AgP group there were only two subjects with a smoking history of <1 year, while there were no smokers in the healthy group. Exclusion criteria were patients with a history of periodontal treatment within the previous 12 months, affected by any systemic condition (including pregnancy), or who were taking any medication that might have the same effect.

The control group was recruited from age- and gender-matched healthy subjects of the general population residing in the same geographic area as G-AgP patients. Full-mouth periodontal examinations were performed on the control subjects. Controls exhibited no signs of periodontal disease as determined by the absence of CAL and no sites with probing depth >3 mm. The study protocol was approved by the Ethics Committee of the Fourth Military Medical University. Informed consent was obtained from all participants in accordance with the Helsinki declaration.

Swab sample collection and DNA preparation

Genomic DNA was extracted from oral mucosa swab sample of the study subjects by the Chelex-100 (Sigma, St. Louis, MO, USA) method. To collect a sample, a swab was scraped with a cotton stick against the inside of each cheek for 30 s. After drying at room temperature for 2 h, the swab coated on the cotton stick was cut from the stick with sterilized scissors. Three hundred microlitres of 5% Chelex 100 (Sigma) and 10 μl of 10 mg/ml proteinase K were added to a cotton swab placed in a 1.5 ml microcentrifuge tube and mixed well. The solution was incubated at 55°C for 30 min. and vortexed. The mixture was incubated in a boiling-water bath for 8 min. and placed in a clean 1.5 ml microcentrifuge tube. After centrifugation at $6000 \times g$ for 5 min., the supernatant of the collection tube was transferred to another 1.5 ml microcentrifuge tube and used for PCR amplification.

Genotype determination

MMP-2 genotypes were determined by PCR-based denaturing high-performance liquid chromatography (DHPLC) analysis while MMP-9 and TIMP-2 genotypes were identified by a PCR-based restriction fragment length polymorphism described previously (Lin et al. 2004, Zhou et al. 2004, Matsumura et al. 2005). All primer sequences for each examined polymorphism, PCR condition and restriction enzyme are shown in Table 1. PCR reaction was accomplished in a final volume of 30 μl, containing ~ 100 ng genomic DNA, 1.0 μM concentration of each primer, 0.2 mM dNTP, 2.0 mM $MgCl_2$, 1.0 U Taq DNA polymerase with $1\times$ reaction buffer (TianGenBiotech Co. Ltd., Beijing, China).

MMP-2 genotyping

DHPLC analysis was performed on a Transgenomic WAVE System (Transgenomic, Inc., Beijing, China). Briefly, each polymerase chain reaction (PCR) product was applied to the DHPLC column, denatured for 5 min. at 95°C and then gradually cooled to 45°C over 30 min. to form homo- and/or heteroduplexes. PCR products were eluted with a linear acetonitrile gradient at a flow rate of 0.9 ml/min. The genotypes

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Table 1. The primer sequences, PCR cycling conditions and restriction enzymes

Polymorphisms	Primer sequences and PCR condition
MMP-2 −1306C/T	5′-CTGACCCCCAGTCCTATCTGCC-3′ 5′-TGTTGGGAACGCCTGACTTCAG-3′ 94°C, 4 min., 35 cycles; 94°C, 30 s; 56°C, 40 s; 72°C, 45 s PCR product for DHPLC analysis
MMP-9 −156-2C/T	5′-GCCTGGCACATAGTAGGCCC-3′ 5′-CTTCCTAGC CAGCCGGCATC-3′ 94°C, 4 min., 35 cycles; 94°C, 1 min.; 68°C, 1 min.; 72°C, 1 min. *Sph*I enzyme digest
TIMP-2 −418-G/C	5′-CGTCTCTTGTTGGCTGGTCA-3′ 5′-CCTTCAGCTCGACTCTGGAG-3′ 94°C, 4 min., 35 cycles; 94°C, 1 min.; 56°C, 45 s; 72°C, 45 s *BsoB*I enzyme digest

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; DHPLC, denaturing high-performance liquid chromatography.

Table 2. General data and clinical parameters of G-AgP patients and controls

Subjects	*n*	Gender: male/female	Age (years)*	PPD (mm)*	CAL (mm)*
G-AgP patients	79	34/45	27.30 ± 5.7	4.1 ± 0.9	5.1 ± 1.0
Controls	128	61/67	28.46 ± 4.9	2.2 ± 0.4	–

*Mean values ± SD.

G-AgP, generalized aggressive periodontitis; PPD, probing pocket depth; CAL, clinical attachment loss.

of MMP-2 −1306C/T revealed the double-peak elution pattern of heterozygous CT-type, whereas the single-peak elution revealed a pattern of homozygous genotypes. A second DHPLC was performed on homozygous samples mixed with a known sequencing-confirmed reference (homozygous CC genotype) sample to generate a heterozygous elution pattern. In this way, the homozygous TT genotype can be determined by DHPLC analysis, which was further confirmed by DNA sequencing.

MMP-9 genotyping

Each PCR product was digested with three units of *SphI* (MBI, Fermentas, Vilnius, Lithuania) overnight and the fragments separated on a 2.5% agarose gel stained with ethidium bromide. The PCR product was 435 bp in length. The C allele was not cut and the T allele was cut into fragments of 247 and 188 bp.

TIMP-2 genotyping

Products were digested with three units of *BsoBI* (New England BioLabs, Beverly, MA, USA) overnight and separated on a 2.5% agarose gel stained with ethidium bromide. The G allele had two *BsoBl* restriction sites and resulted in 230, 51 and 23 bp bands whereas the C allele lacked one *BsoBI* restriction site and thus produced two fragments of 253 and 51 bp.

Quality control

To ensure the impartiality of the genotyping results, all genotyping was performed without knowledge of the subjects' status. Fifteen percentage masked, random samples from the patients and the controls were tested twice by different examiners; the results were concordant for all masked duplicate sets. To confirm the results, representative gel-purified PCR products were purified and directly sequenced by an ABI PRISM 377 DNA analyzer (Perkin-Elmer, Foster City, CA, USA). Sequence results were fitted with a published sequence from NCBI Genome.

Statistical analysis

We performed power calculations to evaluate the sample sizes of the G-AgP and the controls using the statistical program Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA). The level of clinical relevance for the mutant genes was defined as an odds ratio (OR) of 2.5. As no data are available for three analysed polymorphisms in Chinese periodontitis population, we used the published mutant allele frequencies of healthy Chinese as a parameter (Zhou et al. 2004). Differences in the distributions of genotype and allele frequencies between G-AgP patients and controls were analysed by using χ^2 test after Yates' correction (χ^2). The risk associated with individual genotype and allele was calculated as the OR with their 95% confidence interval (95% CI). Analysis was performed using the SPSS 11.0 statistical package (SPSS Inc., Chicago, IL, USA). Deviation from Hardy–Weinberg equilibrium was assessed by goodness-of-fit between the observed and expected numbers using χ^2 test with 1 degree of freedom. All tests were two-sided and statistical significance was assumed at $p<0.05$. Corrected p values were calculated for multiple testing by the Bonferroni method. If three separate comparisons had been made, then the corrected significance level would have been 0.017.

Results

All subjects' profiles were shown by general data and clinical parameters in the G-AgP patients and the healthy controls (Table 2). Two groups were comparable in terms of gender and age ($p = 0.157$ and 0.124, respectively). The mean ages of the patients and the controls were 27.3 and 28.5 years old, respectively.

The distribution of MMP-2, MMP-9 and TIMP-2 genotype in the G-AgP patients and the controls was in accordance with Hardy–Weinberg equilibrium by χ^2 test ($\chi^2 = 0.001$–0.83, $p>0.05$). The sample size for each group was calculated on the basis of the ratio 1.6 of controls to patients, significance level of 0.05 and a power of 75%. The power calculations performed for this study show that the sample size required to ascertain the significance of association of G-AgP to the studied genetic polymorphisms was 78 for G-AgP patients and 117 for controls. This showed that our sample size was large enough to detect association with an acceptable level of confidence.

The genotype distributions and the allele frequencies of MMP-2, MMP-9 and TIMP-2 polymorphisms in the G-AgP patients and the controls are shown in Table 3. In the patients and the controls, the most common genotype of MMP-2 and MMP-9 gene was CC, while the TT homozygote patients were very rare. No significant difference was observed in the distributions of the genotypes and alleles in MMP-2 and

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Table 3. Genotype distributions and allele frequencies of MMP-2, MMP-9 and TIMP-2 in G-AgP patients and controls

Genotypes	G-AgP patients, $n = 79$ (%)	Controls, $n = 128$ (%)	G-AgP *versus* controls		Alleles	G-AgP patients, $n = 158$ (%)	Controls, $n = 256$(%)	G-AgP *versus* controls	
			OR (95% CI)	*p* value				OR (95% CI)	*p value*
MMP-2CC									
CC	63 (79.7)	98 (76.6)	0.83 (0.42–1.65)	0.716*	C	141 (89.2)	224 (87.5)	0.84 (0.45–1.58)	0.707
CT	15 (19.0)	28 (21.9)			T	17 (10.8)	32 (12.5)		
TT	1 (1.3)	2 (1.5)							
MMP-9									
CC	62 (78.5)	101(78.9)	1.03 (0.52–2.03)	1.000*	C	139 (88.0)	228 (89.1)	1.11 (0.60–2.07)	0.858
CT	15 (19.0)	26 (20.3)			T	19 (12.0)	28 (10.9)		
TT	2 (2.5)	1 (0.8)							
TIMP-2									
GG	41 (51.9)	87 (68.0)	1.97 (1.11–3.50)	0.030†	G	113 (71.5)	211 (82.4)	**1.87 (1.17–2.99)**	**0.013**
GC	31 (39.2)	37 (28.9)			C	45 (28.5)	45 (17.6)		
CC	7 (8.9)	4 (3.1)							

*CT+TT *versus* CC.

†GC+CC *versus* GG; adjusted significance level *p* value is 0.017.

Bold characters indicate statistically significant data.

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; G-AgP, generalized aggressive periodontitis; OR, odds ratio; CI, confidence interval; *p value*, χ^2 test after Yates' correction.

MMP-9 between the patients and the controls ($p > 0.05$).

The homozygotes and heterozygote of TIMP-2 appeared to be distributed evenly in the two groups. Yates' correction showed a marked difference of the distribution of TIMP-2GC and CC genotypes in the groups [$p = 0.030$; OR: 1.97, 95% CI: (1.11, 3.50)], which would not be significant after adjusted $p-$values level for multiple comparison. The frequencies of the alleles TIMP-2 -418G and -418C were 71.5% and 28.5% in the patients and 82.4% and 17.6% in the controls, respectively. A significant increase occured in the allele C of the patients compared with the controls [$p = 0.013$; OR: 1.87, 95% CI: (1.17, 2.99)].

TIMP-2 is considered a specific inhibitor of MMP-2; therefore the analyses of a potential composite genotype were carried out (data not shown). Because the MMP-2 -1306TT and TIMP-2 -418CC homozygotes were rare in this study, these genotypes were, respectively, combined with the MMP-2 -1306CT and TIMP-2 -418GC heterozygotes for estimation of susceptibility to G-AgP. Using the most common genotype MMP-2CC and TIMP-2GG as a reference, however, no significant synergistic effect on the occurrence of G-AgP was observed in subjects carrying the composite genotypes of MMP-2 and TIMP-2 variants ($p > 0.05$).

Discussion

Host-derived MMPs are thought to play a prominent role in the tissue destruction in the progression of periodontitis (Korostoff et al. 2000). The elevated level of MMPs released from host cells not only may be due to the stimulation of pro-inflammatory cytokines and bacteria but also may be dependent on the host genetic polymorphisms in MMPs/TIMPs genes. We selected MMP-2, MMP-9 and TIMP-2 in this study as candidate genes because their protein products express differently in GCF and gingival tissue during the periodontitis process (Makela et al. 1994, Nomura et al. 1998).

In the present study, frequencies of the CC homozygosis of the MMP-2 and MMP-9 gene polymorphisms were higher in the Chinese population than that in Caucasian (51.97%) and Brazilian subjects (63.2%), whereas allele T frequencies were lower in Chinese than the Caucasian (26.4%) and Brazilian (19.7%) (de Souza et al. 2005, Holla et al. 2005). TIMP-2 -418G/C genotype also differs from previous results in the Brazilian population (de Souza et al. 2005). The present data failed to demonstrate a relationship between MMP-2, MMP-9 polymorphisms and G-AgP, which was not in agreement with recent studies on CP (Holla et al. 2006, Keles et al. 2006). A possible explanation for these conflicting findings may lie in the distinct race having different risk alleles for a different phenotype. Therefore, possible positive associations between a genetic marker and disease within one population may not necessarily be extrapolated to other populations (Loos et al. 2005). In addition, at low frequency of the allele even having a relatively strong effect, this allele can be difficult to record in a population. G-AgP is a complex disease with large heritable components. To identify with confidence an association with such small effects for these genes, very large numbers of patients will need to be studied (Taylor et al. 2004).

In this study, we found that the distribution of mutant gene C carriers in TIMP-2 was statistically different between the patients and the controls. Statistical analyses indicated that individuals harbouring GC/CC genotypes had an increasing risk of developing G-AgP ($p = 0.030$). To compensate for multiple testing, we utilized the Bonferroni correction, which caused the significant *p* value ($p > 0.017$) to disappear. However, a significant difference of the frequencies in TIMP-2 -418G and C allele was also observed in two groups ($p = 0.013$). These findings imply that functional polymorphism in TIMP-2 -418G/C affects susceptibility of G-AgP in the Chinese subjects.

Furthermore, genetically complex diseases differ from simple Mendelian diseases. These complex traits are the result of the interaction of alleles at multiple different gene loci. However, we could not find any significant difference of combined MMP-2 and TIMP-2 genotype frequencies in two groups. The association between polymorphism and susceptibility of periodontitis should therefore be interpreted with caution (Kinane & Hart 2003, Taylor et al. 2004, Loos et al. 2005).

In conclusion, we investigated three functional polymorphisms in MMP-2,

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MMP-9 and TIMP-2 candidate genes for G-AgP and found an association between allele G/C in TIMP-2 −418 and G-AgP. The increased frequencies of C allele presented in G-AgP may be unfavourable.

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Clinical Relevance

Scientific rationale for the study: AgP has a genetic basis. Host-derived MMPs and TIMPs contribute to the destruction of supporting tissue in periodontitis. Polymorphisms identified in MMP-2, MMP-9 and TIMP-2 genes influence transcriptional activity level. The study was carried out to analyse the genotypes in Chinese patients with G-AgP.

Principal findings: The analysis of the TIMP-2 −418G to C gene polymorphism revealed significant differences between the patients and controls. Compared with the controls, a significant increasing trend occurred in the genotype GC/CC and allele C of G-AgP patients.

Practical implications: The carriers of TIMP-2 −418C polymorphism in the Chinese subjects may be more at risk of suffering from G-AgP.

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