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Effect of matrix metalloproteinase-1 promoter genotype on interleukin-1beta-induced matrix metalloproteinase-1 production in human periodontal ligament cells

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Background and Objective: Our previous studies have shown that a single nucleotide polymorphism at nucleotide −1607 of the human matrix metalloproteinase-1 (MMP-1) promoter is associated with the severity of periodontal disease. The purpose of this study was to evaluate the effect of different MMP-1 promoter genotypes on interleukin-1beta-induced MMP-1 production in human periodontal ligament cells.

Material and Methods: Periodontal ligament cells from 16 different subjects were cultured. Restriction fragment length polymorphism–polymerase chain reaction was used to identify the MMP-1 promoter genotype of periodontal ligament cells. Periodontal ligament cells were stimulated with phorbol 12-myristate 13-acetate/interleukin-1beta. Reverse transcription–polymerase chain reaction amplifications and enzyme linked immunosorbent assays were then utilized to determine the MMP-1 mRNA levels and to assess the concentration of MMP-1 protein, respectively.

Results: The results showed that cells with the 1G/2G and the 2G/2G genotype produced higher amounts of MMP-1 protein than cells with the 1G/1G genotype. Induction of MMP-1 mRNA as a result of stimulation with interleukin-1beta was significantly increased in cells with a 1G/2G or a 2G/2G genotype compared with cells homozygous for the 1G allele.

Conclusion: The results of the present study suggest that the single nucleotide polymorphism at nucleotide −1607 of the human MMP-1 promoter might influence the interleukin-1beta-induced expression of MMP-1 in periodontal ligament cells.

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The host response to periodontal infection plays a major role in the pathogenesis of periodontal diseases (1). Combinations of bacteria and of their products, and responses of the host to these bacteria and to their products, determine the intensity of the inflammatory reaction (1–3), which is mediated by the immune system. Corresponding histopathological changes include the accumulation of inflammatory cells in the gingival tissues, vascular and epithelial changes, and the degradation of collagen (4).

Bacteria are essential for the initiation of periodontitis, but host factors are largely responsible for the development of a chronic inflammatory state, leading to destruction of the periodontal support structure (5). Periodontitis is characterized by the degradation of the extracellular matrix of periodontal tissue. Matrix metalloproteinase-1 (MMP-1) is the key proteolytic enzyme responsible for cleaving native interstitial type I and type III collagens, which are the most abundant protein components of the extracellular matrix in the periodontium. MMP-1 serves as an initiator of extracellular matrix destruction and co-operates with other MMPs in the degradation of collagen. Moreover, the concentration of MMP-1 is elevated in gingival crevicular fluid and in periodontal tissue in association with periodontitis (6).

A genetic variation in the MMP-1 promoter region can influence the transcription of MMP-1, and hence this gene might be crucial in mediating connective tissue degradation in the pathogenesis of periodontitis. The genetic variation responsible for influencing the transcription of MMP-1 is a single nucleotide polymorphism located at nucleotide −1607, where an additional guanine creates an Ets-binding site, namely 5′-GGA-3′, instead of 5′-GAT-3′, the core binding site for members of the Ets transcription factors (7). It has been shown that the 2G allele can significantly increase the transcription activity of MMP-1 as compared with the 1G allele (7). Holla *et al.* (8) recently demonstrated that the association between the 1G/2G polymorphism of MMP-1 and chronic periodontitis was observed strongly in nonsmokers but not in smokers, and the polymorphisms in the MMP-1 promoter may have a limited effect on the etiopathogenesis of chronic periodontitis. Our previous study also substantiated that the 1G/2G polymorphism could be a risk factor for generalized aggressive periodontitis and chronic periodontitis in Chinese people (9,10).

MMP-1 has been found to be the primary collagenase involved in interstitial collagen degradation, and its concentration in gingival crevicular fluid is increased in association with periodontitis. Enhanced levels of MMP-1 protein mRNA, were also detected in patients with periodontitis (11,12). As a marker of active inflammation, interleukin-1 may contribute to the pathologic process of the inflammatory response (13,14). Interleukin-1 occurs in two forms – interleukin-1alpha and interleukin-1beta – of which interleukin-1beta appears to be the most potent form, having a catabolic effect on bone which is approximately 10-fold higher than that of interleukin-1alpha. The concentration of interleukin-1beta has been found to be significantly increased in periodontal tissues and gingival fluid from diseased sites, compared with periodontal tissues and gingival fluid from healthy sites (15). Interleukin-1beta can also induce the expression of MMPs in periodontal ligament cells (16,17).

In the present study, we will confirm if the polymorphism at nucleotide −1607 of the MMP-1 promoter indeed contributes to the regulation of endogenous MMP-1 gene transcription and to the level of MMP-1 produced in interleukin-1beta-stimulated human periodontal ligament cells.

Material and methods

Cell culture

Human periodontal ligament cells were isolated from the first premolar teeth of individuals undergoing tooth extraction for orthodontic treatment in accordance with the method of Somerman *et al.* (18), with minor modifications. All subjects were in good general health and had a healthy periodontal condition. The probing depths were < 3 mm, and there was no reduction of periodontal attachment level. Healthy periodontal tissue was removed from the center of the root surface using a surgical scalpel. The tissue was minced and then transferred to a culture flask. The explants were cultured in Dulbecco's modified Eagle's minimal essential medium (Gibco BBL, New York, NY, USA) supplemented with 10% fetal bovine serum (Gibco BBL), 100 units/mL of penicillin G and 100 μg/mL of streptomycin, with a change of medium every 2 or 3 d. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When the cells growing out from the explants had reached confluence, they were separated by treatment with 0.25% trypsin/0.53 mM EDTA, collected by centrifugation and cultured on plastic culture dishes containing standard medium (Dulbeccos modified Eagles minimal essential medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin G and 100 lg/mL of streptomycin) until reaching confluence. The cells were then trypsinized at 1:3 split ratios. Experiments were carried out on cells from the fourth to fifth passages.

The study was authorized by the Research Ethics Committee of School & Hospital of Stomatology, Wuhan University. Each subject was promised that their donated periodontal ligament cells would be utilized only for this research, not for any other biological research, including human cloning and tissue engineering. All patients gave informed consent before providing the periodontal ligament cell samples. The patients were also reassured that any information obtained in connection with the study that could be identified as originating from the patient would remain confidential and be disclosed only with their permission. The volunteers were free to discontinue participation at any time without prejudice.

In this study, we established 16 cell lines from different volunteers, and all cell lines were genotyped using restriction fragment length polymorphism–polymerase chain reaction (RFLP–PCR) methodology.

Genome DNA samples from human periodontal ligament cells

DNA was extracted from cultured periodontal ligament cells using a genetic extraction kit [Takara Biotechnology (Dalian) Co. Ltd, Dalian, China]. DNA was dissolved in 10 mM Tris (pH 7.8)/1 mM EDTA buffer. The DNA concentration was estimated by measuring the absorbance at 260 nm. The final preparation was stored at −20°C until further analysis.

MMP-1 promoter genotyping of human periodontal ligament cells

The sequences of the PCR primers were as follows: forward, 5′-TCGTGAGAATGTCTTCCCATT-3′; and reverse, 5′-TCTTGGATTGATTTGAGATAAGTGAAATC-3′. The reverse primer was specially designed to introduce a recognition site for the restriction enzyme, *Xmn*I, according to the protocol employed in previous studies (12). The 1G allele has this recognition site, whereas the 2G allele does not have this recognition site because of the insertion of a guanine (G). PCR amplification was carried out in a total volume of 25 μL, containing 100 ng of genomic DNA, 10 mM Tris–HCL (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 pM each primer and 2 units of Taq DNA polymerase (Biostar International, Toronto, ON, Canada). The PCR cycling conditions were: 1 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, with a final extension at 72°C for 5 min.

A 15 μL aliquot of PCR products was mixed with 4 μL of solution containing 2 μL of 10× RE buffer, 2 μL of bovine serum albumin (10 mg/mL) and 1 μL of *Xmn*I (10 U/μL; Promega, San Luis Obispo, CA, USA). This sample, containing restriction endonuclease, was incubated at 37°C overnight and then mixed with 2 μL of loading buffer. The sample was electrophoresed on a 12% vertical nondenaturing polyacrylamide gel at 50 V for 4 h and the DNA bands were visualized following staining with ethidium bromide.

MMP-1 protein analysis

Periodontal ligament cells with different genotypes were seeded into 24-well plastic culture plates (1×10^5 cells/well) and cultured in Dulbecco's modified Eagle's minimal essential medium, supplemented with 10% fetal bovine serum, to reach 90% confluence. Cells were then incubated for 24 h with 1 ng/mL of interleukin-1beta or with 100 pmol/mL of phorbol 12-myristate 13-acetate (phorbol 12-myristate 13-acetate is a protein kinase C activator) in Dulbecco's modified Eagle's minimal essential medium supplemented with 1% fetal bovine serum. Conditioned medium was then collected and supplemented with a protease inhibitor solution (Roche, Indianapolis, IN, USA), at a ratio of 9:1 (v/v), then rapidly transferred into liquid nitrogen and stored at −70°C. The concentrations of MMP-1 were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Minneapolis, MN, USA), as described by the manufacturer. Each sample was tested in duplicate.

RNA isolation

Periodontal ligament cells with different genotypes were incubated for 24 h with 1 ng/mL of interleukin-1beta or 100 pmol/mL of phorbol 12-myristate 13-acetate in Dulbecco's modified Eagle's minimal essential medium supplemented with 1% fetal bovine serum. Total RNA was extracted from human periodontal ligament cells using the total RNA extract kit [RNAiso Reagent; Takara Biotechnology (Dalian) Co. Ltd], according to the manufacturer's protocol. RNA concentrations were calculated from the absorbance at 260 nm, and purity was assessed by the 260 : 280 absorbance ratio. Total RNA was stored at −70°C before analysis by RT-PCR.

RT-PCR

Specific mRNA was reversed transcribed into cDNA by avian myeloblastosis virus reverse transcriptase (Takara Biotechnology Co. Ltd, Otsu, Shiga, (as per google) Japan) in the presence of oligo (dT) primer, with 1.0 μg of total RNA being used for the reaction. The PCR amplification reaction utilized Taq polymerase and the following specific primers for MMP-1: forward primer, 5′-CATCCAAGCCATATATGGACGTTCC-3′; and reverse primer, 5′-TCTGGAGAGTCAAAATTCTCTTCGT-3′. The product size was 611 bp. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel, visualized by staining with ethidium bromide and analyzed densitometrically using the Gene Genius Bio imaging system (Syngene, Frederick, MD, USA). The relative levels of mRNA expression were quantified by comparison with the internal control (beta-actin: forward primer 5′-GCGAGAAGATGACCCAGATCATGTT-3′ and reverse primer 5′-GCTTCTCCTTAATGTCACGCACGAT-3′; the product size was 300 bp). Each PCR amplification was carried out in duplicate using the same cDNA. The PCR was performed over 28 cycles (5 min at 94°C, 45 s at 94°C, 45 s at 55°C, 1 min at 72°C, followed by a 7 min extension at 72°C).

Statistical analysis

The differences among cells with different genotypes with respect to MMP-1 mRNA and protein expression were examined using analysis of variance. p-Values of < 0.05 were considered statistically significant.

Results

Genotype identification of the periodontal ligament cells

Two mismatches were introduced into the reverse primer annealed sequence (5′-GAANNNNTTC-3′) to create a restriction site for the restriction endonuclease *Xmn*I. Thus, *Xmn*I digested the 1G allele, creating two fragments of 89 and 29 bp. Hardy–Weinberg equilibrium is a basic principle of population genetics. Basically, the frequency of the homozygote is almost the square of the frequency of the allele. This polymorphism also demonstrated Hardy–Weinberg equilibrium. The MMP-1 promoter single nucleotide polymorphism of periodontal ligament cells

Table 1. The data of the volunteers and corresponding periodontal ligament cell genotypes

		Age (years)	Gender	
Genotype	*n*	Mean ± SD	Male	Female
2G homozygote	5	17.8 ± 4.76	2	3
Heterozygote	7	17.1 ± 5.01	4	3
1G homozygote	4	18.8 ± 5.16	2	2

consisted of the 2G homozygote (no. 5), the 1G/2G heterozygote (no. 7) and the 1G homozygote (no. 4). The periodontal ligament cells were divided into three groups according to genotype. The data of the volunteers and of the corresponding periodontal ligament cell genotypes are summarized in Table 1.

Influence of MMP-1 promoter genotype on interleukin-1beta-induced MMP-1 protein expression

Both phorbol 12-myristate 13-acetate and interleukin-1beta were able to induce the production of MMP-1 in periodontal ligament cells, and the production of MMP-1 protein in response to phorbol 12-myristate 13-acetate/interleukin-1beta was also influenced by the MMP-1 promoter genotype (Fig. 1). When periodontal ligament cells were incubated with phorbol 12-myristate 13-acetate, the MMP-1 production level, compared with the control, in cells with the 2G/2G genotype was 2.59-fold higher than in cells with the 1G/1G genotype. For cells with the 1G/2G genotype, the increased level of MMP-1 production was 2.26-fold higher than for cells with the 1G/1G genotype ($p = 0.00 < 0.05$). When periodontal ligament cells were incubated with interleukin-1beta, the MMP-1 concentration was 7.75-fold higher in cells with the 2G/2G genotype than in cells with the 1G/1G genotype, and 5.13-fold higher in cells with the 1G/2G genotype than in cells with the 1G/1G genotype ($p = 0.00 < 0.05$). Besides, the relative level of MMP-1 protein in periodontal ligament cells with the 2G/2G genotype was also significantly higher than in periodontal ligament cells with the 1G/2G genotype ($p = 0.00 < 0.05$).

Periodontal ligament cells with the 2G allele showed a greater MMP-1 response to stimulation with phorbol 12-myristate 13-acetate/interleukin-1beta than cells homozygous for the 1G allele, in terms of MMP-1 detected by the absolute amount of MMP-1 elaborated, as determined using ELISA.

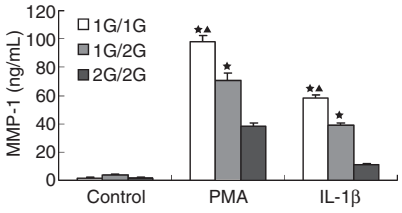

Fig. 1. The concentration of matrix metalloproteinase-1 (MMP-1) protein in periodontal ligament cells with different −1607 MMP-1 promoter genotypes, in response to stimulation with phorbol 12-myristate 13-acetate or interleukin-1beta. Quantitative analysis of MMP-1 protein content was carried out on periodontal ligament cells with the indicated MMP-1 promoter genotypes. Cells were cultured for 24 h with 100 pmol/mL of phorbol 12-myristate 13-acetate or with 1 ng/mL of interleukin-1beta. Values are the means ± standard deviation. From the indicated number of different cell preparations, MMP-1 production by each cell preparation was determined in triplicate cultures. Values with a different symbol are significantly different (analysis of variance). ★, significant differences ($p = 0.00 < 0.05$) compared with the 1G/1G group; ▲, compared with the 1G/2G group ($p = 0.00 < 0.05$), the presence of the 2G allele may significantly increase the induction of MMP-1 protein following stimulation with phorbol 12-myristate 13-acetate. IL-1β, interleukin-1beta; PMA, phorbol 12-myristate 13-acetate.

Influence of MMP-1 promoter genotype on induction of MMP-1 mRNA

RT-PCR was performed to assess the induction of MMP-1 mRNA in periodontal ligament cells with different MMP-1 promoter genotypes in response to stimulation with phorbol 12-myristate 13-acetate/interleukin-1beta. The relative levels of MMP-1 mRNA expression were quantified by comparison with the internal control (beta-actin). Cells with the 1G/2G ($p = 0.004 < 0.05$) and 2G/2G ($p = 0.00 < 0.05$) genotypes showed significantly stronger induction of MMP-1 mRNA than cells with the 1G/1G genotype. Besides, the relative level of MMP-1 mRNA expression in periodontal ligament cells with the 2G/2G genotype was also significantly stronger than that of periodontal ligament cells with the 1G/2G genotype ($p = 0.00 < 0.05$) (Figs 2 and 3).

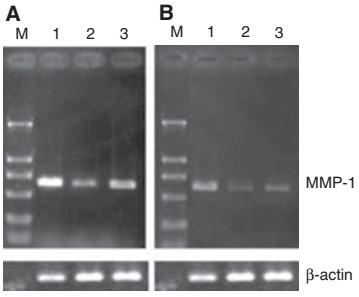

Fig. 2. Influence of matrix metalloproteinase-1 (MMP-1) promoter genotype on the induction of MMP-1 mRNA in periodontal ligament cells, as determined using semiquantitative reverse transcription–polymerase chain reaction analysis. (A) Periodontal ligament cells with different MMP-1 promoter genotypes were treated with 100 pmol/mL of phorbol 12-myristate 13-acetate for 24 h. (B) Periodontal ligament cells with different MMP-1 promoter genotypes were treated with 1 ng/mL of interleukin-1beta for 24 h. (M, DNA maker; 1, 2G/2G genotype; 2 , 1G/1G genotype; 3, 1G/2G genotype). β-actin, beta actin.

Discussion

MMPs are a family of metal-dependent proteolytic enzymes that mediate the degradation of extracellular matrix and basement membranes. Among the MMPs, MMP-1 is a highly expressed interstitial collagenase, responsible for degrading fibrillar collagens, the most abundant protein in the periodontal tissue. Rutter *et al.* (19) found that insertion of a guanine base (G) creates the sequence 5′-GGAT-3′, the core

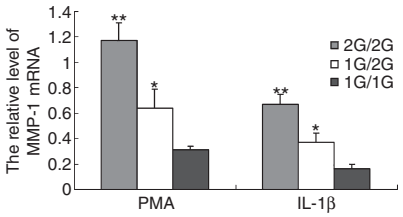

Fig. 3. The level of matrix metalloproteinase-1 (MMP-1) mRNA expression in periodontal ligament cells with different −1607 MMP-1 promoter genotypes in response to stimulation with phorbol 12-myristate 13-acetate/interleukin-1beta. Quantitative analysis of MMP-1 mRNA expression was carried out on periodontal ligament cells with the indicated MMP-1 promoter genotype. Cells were cultured for 24 h with 100 pmol/mL of phorbol 12-myristate 13-acetate or with 1 ng/mL of interleukin-1beta. Values are the means ± standard deviation. From the indicated number of different cell preparations, the relative level of MMP-1 mRNA produced by each cell preparation was determined quantitatively using densitometry, which was compared with the internal control (beta-actin) in triplicate cultures. Values with a different symbol are significantly different by analysis of variance. **, significant differences ($p = 0.00 < 0.05$) compared with the 1G/1G group; *, compared with the 1G/1G group ($p = 0.004 < 0.05$), the 2G allele may significantly increase the induction of MMP-1. IL-1β, interleukin-1beta; PMA, phorbol 12-myristate 13-acetate.

binding site for members of the Ets family of transcription factors, and increases the promoter activity in transfected cells. It has been demonstrated that the 2G allele displays heightened transcription of MMP-1 in both tumor cells and normal fibroblasts, and these heightened levels of MMP-1 expression may result from the presence of the 2G allele and from elevated expression of the transcription factors that bind to this site. The presence of this allele has been associated with the development of ovarian cancer and breast cancer (7,20).

Our previous study also substantiated that the 1G/2G polymorphism was associated with generalized aggressive periodontitis and chronic periodontitis in Chinese people (9,10), which was consistent with the results of other studies (8,21). However, interestingly, Holla *et al.* (8) found that when only nonsmoking subjects were included in the analysis, the difference in the allelic frequencies of the 1G/2G polymorphism between the chronic periodontitis patients and the control population became significant. Brazilians with the 2G allele seem to have a greater risk for developing severe chronic periodontitis (21). Our result seems not to be in agreement with reports on Japanese people (22). As mentioned above, the distribution of genotypes is often different in different races. This discrepancy may be explained by the fact that we analyzed the genotype distribution and the allele frequency in Chinese people.

For the first time, we also confirmed a direct correlation between the MMP-1 polymorphism and the transcription of MMP-1 in periodontal ligament cells. In this study, because phorbol 12-myristate 13-acetate is a protein kinase C activator and could induce the expression and secretion of MMPs in many cells, it was considered to be a positive control of interleukin-1beta. Many growth factors could regulate the expression of MMPs (23), and Nakaya *et al.* (16,17) observed that interleukin-beta might up-regulate the expression of MMP-1 and MMP-3 in human periodontal ligament cells. When periodontal ligament cells with different genotypes were treated with phorbol 12-myristate 13-acetate/interleukin-1beta, periodontal ligament cells with a 1G/2G genotype or a 2G/2G genotype showed a greater response, in terms of MMP-1 production (determined by ELISA as the absolute amount of MMP-1 produced), to stimulation with phorbol 12-myristate 13-acetate/interleukin-1beta than cells homozygous for the 1G allele. Furthermore, cells with the 2G/2G genotype produced significantly higher amounts of MMP-1 compared to cells with the 1G/2G genotype, and when periodontal ligament cells were treated with phorbol 12-myristate 13-acetate/interleukin-1beta, cells with 1G/2G and 2G/2G genotypes also produced a significantly higher amount of MMP-1 than cells with the 1G/1G genotype.

Because the production of MMP-1 transcripts and protein correlated with the genotypes of periodontal ligament cells, we concluded that the 2G allele exerts a dominant effect, rendering periodontal ligament cells significantly more responsive to phorbol 12-myristate 13-acetate/interleukin-1beta-induced MMP-1 expression. This assumption was highly compatible with the finding reported by Fujimoto (24), that phorbol 12-myristate 13-acetate increased amnion mesenchymal cell nuclear protein binding with greater affinity to the 2G allele, and concluded that the 2G allele had stronger promoter activity in amnion cells. Therefore, the amnion cells with the 2G allele had increased responsiveness to stimuli that induce MMP-1. This polymorphism contributed to the risk of preterm premature rupture of the membranes.

Wyatt's (25) study showed that the mean level of MMP-1 expression in epidermal growth factor-stimulated 2G homozygous human foreskin fibroblasts was higher than that in 2G homozygous cells. Our findings suggested that the functional differences of the two MMP-1 promoter alleles might become apparent when periodontal ligament cells are challenged with agents that promote MMP expression. Another study in our research group showed that the induction of MMP-1 by centrifugal force was significantly greater in cells with a 2G/2G genotype or a 1G/2G genotype than in cells homozygous for the 1G allele. The MMP-1 mRNA and protein levels were significantly higher in cells with the 2G allele than in cells with the 1G/2G allele or the 1G allele (26).

Therefore, these differences observed in the generation of MMP-1 by two different MMP-1 promoter alleles indicate that the MMP-1 genotype might have an impact *in vivo* under specific environmental conditions in which cells are exposed to MMP-1 inducing stimuli (e.g. infection or inflammation) (24). As other MMPs also participate in both the activation of pro-MMP-1 and the further catabolism of collagen fragments, variants in other MMP genes may augment or attenuate the influence of the MMP-1 promoter polymorphism. It will therefore be important to explore, in the

future, the relationship between the MMP-1 promoter genotype and other environmental variables. In further research, the difference in response to treatment of periodontitis patients with different MMP-1 promoter genotypes, and their prognosis diversity, should be investigated. Pirhan *et al*. (27) carried out the first study to investigate the effect of polymorphisms at the −1607 nucleotide of the MMP-1 gene promoter on the clinical status and the gingival crevicular fluid MMP-1 levels, as well as on the nonsurgical periodontal treatment outcomes, in patients with chronic periodontitis. A higher percentage of sites with clinical attachment level of 4–6 mm, together with knowledge of the gingival crevicular fluid MMP-1 levels in the MMP-1 2G allele carriers at baseline and after nonsurgical therapy, might draw attention to the importance of regular maintenance in these patients for the prognosis of periodontal disease progression following periodontal therapy. The study indicated that more extensive studies with larger patient groups and also with other ethnic populations should be undertaken in order to analyze the putative relevance of MMP-1 promoter gene polymorphisms at the −1607 nucleotide in the pathogenesis of periodontitis, which would also be valuable in preventive, diagnostic and therapeutic strategies against the development of periodontitis.

In conclusion, our present study reported the novel finding that the polymorphism at the −1607 nucleotide of the MMP-1 promoter had functional significance in controlling the level of transcription and of protein production of MMP-1 in human periodontal ligament cells *in vitro*, which implies that periodontitis subjects with the 2G allele might experience more severe degradation of the periodontal extracellular matrix *in vivo*.

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