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***MMP3* and *TIMP1* variants contribute to chronic periodontitis and may be implicated in disease progression**

Ariadne Letra, D.D.S., M.S., Ph.D.^{a,1}, Renato M. Silva, D.D.S., M.S., Ph.D.^a, Ryan J. Rylands, B.S.^b, Elcia Maria Silveira, D.D.S., M.S.^c, Ana Paula de Souza, D.D.S., M.S., Ph.D.^d, Steven K. Wendell, Ph.D.^b, Gustavo P. Garlet, D.D.S., M.S., Ph.D.^c, and Alexandre R. Vieira, D.D.S., M.S., Ph.D.^b

^aDepartment of Endodontics, School of Dentistry, and Pediatric Research Center, Medical School, University of Texas Health Science Center at Houston

^bDepartments of Oral Biology and Center for Craniofacial and Dental Genetics, Pediatric Dentistry, School of Dental Medicine, University of Pittsburgh

^cDepartment of Oral Histology, Bauru Dental School, University of São Paulo, Brazil

^dDepartment of Morphology, Piracicaba School of Dentistry, University of Campinas, Brazil

Abstract

Aim—Matrix metalloproteinases (MMPs) play a key role in the tissue destruction characteristic of chronic periodontitis. The purpose of this study was to investigate the association of MMP and TIMP polymorphisms with chronic periodontitis in two populations.

Materials and Methods—Thirty-four polymorphisms spanning 12 MMP and 2 TIMP genes were genotyped in 401 individuals from Brazil (99 cases with chronic periodontitis and 302 controls), and 274 individuals from the US (70 cases and 204 controls). Individuals were considered cases if presenting at least three teeth exhibiting sites of clinical attachment loss ≥5mm in two different quadrants. Controls were characterized by absence of clinical attachment loss and no sites with probing depth >3mm. *MMP3* and *TIMP1* mRNA expression was evaluated in healthy and diseased periodontal tissues.

Results—*TIMP1* showed association with chronic periodontitis in the Brazilian population (for rs5906435, $P=0.0004$), whereas *MMP3* showed association in the US population (for rs679620, $P=0.0003$; and rs650108, $P=0.002$) and in the Brazilian population (for rs639752, $P=0.005$). *MMP3* and *TIMP1* mRNA expression was significantly higher in diseased tissues when compared to control tissues.

Conclusions—Our results further support a role for variations in *MMP3* in chronic periodontitis and report a novel association with *TIMP1*. These genes may be considered additional candidate genes for chronic periodontitis.

Keywords

periodontitis; MMP; TIMP; polymorphism

Corresponding author: Ariadne Letra, DDS, MS, PhD, Associate Professor, Department of Endodontics, University of Texas Health Science Center at Houston, School of Dentistry, 7500 Cambridge St., Room 6411, Houston, TX 77030, Office phone: 713-486-4228, Fax: 713-486-0402, ariadne.m.lettra@uth.tmc.edu.

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INTRODUCTION

Periodontal diseases are progressive and destructive inflammatory conditions of the tooth-supporting tissues. The disease primarily affects the supporting connective tissues of the tooth, known as periodontal ligaments and alveolar bone, and is a major cause of tooth loss in adults. During this event, the most important component of the periodontium lost is collagen type I, which is found in the periodontal ligament and alveolar bone organic matrix. Periodontal disease is usually termed as aggressive periodontitis, when characterized by rapid localized attachment loss and bone destruction, and usually affects healthy juveniles or early adults by familial aggregation; or as chronic periodontitis, the most frequent type, characterized by slow to moderate rates of progression in areas of frequent presence of plaque and calculus (Armitage 2004). Chronic periodontitis is initiated and maintained by a complex microbial biofilm that forms on the surfaces of teeth in close proximity to their supporting tissues, however, a wide variety of other determinants including demographic, social, behavioral, environmental, and systemic factors as well as genetic factors have been identified to affect the distribution, severity, and extension of the disease (Nunn, 2003).

Several lines of evidence suggest that matrix metalloproteinases (MMPs) comprise the most important pathway in the tissue destruction associated with periodontal disease, due to their role in the pathological breakdown of extracellular matrix within the periodontal tissues (Hannas et al., 2007). Together with their endogenous tissue inhibitors of metalloproteinases (TIMPs), MMPs also have a key role in the physiological tissue remodeling of the periodontal tissues, and an imbalance between MMPs and TIMPs has been observed in association with tissue breakdown and periodontal disease (Garlet et al., 2004). Further, active collagenases (MMPs -1, -8, and -13) and gelatinases (MMPs -2, and -9) have been detected in the crevicular gingival fluid (Ingman et al., 1996; Rai et al., 2008; Teng et al., 1992), saliva (Ingman et al., 1996; Makela et al., 1994; Rai et al., 2008), and biopsy specimens of inflamed periodontal tissues (Ejeil et al., 2003; Makela et al., 1994) from patients with periodontitis in much larger amounts than in control subjects.

Studies of polymorphisms in cytokines and MMP genes have provided important clues as to the nature of genetic factors in periodontal disease (Vijayalakshmi et al. 2010). Some of these polymorphisms may result in either altered expression or in functional changes of the encoded molecules, therefore perhaps making individuals with aberrant genotypes more susceptible to a given disease, or resulting in an increase of disease severity. Polymorphisms in the genes encoding MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 have been frequently investigated for association with chronic periodontitis in distinct populations, and the most consistent data relies on the association of a MMP-1 polymorphism characterized by an insertion/deletion of a guanine at position -1607 of the gene promoter (Astolfi et al., 2006; Chou et al., 2011; de Souza et al., 2003; Repeke et al., 2009). Nevertheless, most of these studies reflect investigation of an individual polymorphisms in a single gene or in a couple of genes, which makes interpretation of the results difficult, taken the complexity of the MMP family and their compensatory mechanisms.

In the current study, we investigated 12 MMP and 2 TIMP genes for association with chronic periodontitis in two distinct populations. We also investigated the expression of *MMP3* and *TIMP1* mRNA transcript levels in diseased and healthy periodontal tissues. Variants in *MMP3* and *TIMP1* were associated with chronic periodontitis and their expression levels may be implicated in disease progression status.

MATERIALS AND METHODS

Genetic analysis

Sample Populations—This study was approved by the local and the University of Pittsburgh Institutional Review Boards (Process 0511110) and participants signed an informed consent and provided a saliva sample as source of genomic DNA.

Two case-control datasets from distinct populations were tested for association with MMP and TIMP polymorphisms and chronic periodontitis in this study. The first dataset consisted of 401 individuals, 99 cases with chronic periodontitis (40 males, 59 females, average age 45 ± 11 SD) and 302 controls (108 males, 194 females, average age 42 ± 15 SD) without periodontitis, recruited at the Dental Clinics at Sagrado Coração University, and Bauru Dental School, Bauru, Brazil. This population has been described in part elsewhere (Astolfi et al., 2006; Letra et al., 2007). The second dataset consisted of 274 Caucasian individuals from the US, 70 cases presenting chronic periodontitis (36 males, 34 females, average age 54 ± 10 SD) and 204 controls (89 males, 115 females, average age 50 ± 8 SD). Individual samples and clinical history were obtained through the Dental Registry and DNA Repository of the School of Dental Medicine, University of Pittsburgh.

Clinical and radiographic examinations were performed by two calibrated examiners at each study collection site. Interobserver agreement on the diagnosis of chronic periodontitis was calculated using Kappa statistic ($K=0.92$) as described elsewhere (Viera and Garrett, 2005). Individuals were considered cases if presenting at least three teeth exhibiting sites of clinical attachment loss ≥ 5 mm in two different quadrants. Controls were characterized by absence of clinical attachment loss and no sites with probing depth >3 mm. Details of the studied populations are presented in Table 1.

Selection of Candidate Genes and Single Nucleotide Polymorphisms—We selected 12 MMP and 2 TIMP genes for investigation in this study. We prioritized the genes to be studied considering: a) previous reports of expression in diseased tissues, b) previous reports of association with periodontal disease, and c) substrates as recognized molecules in diseased tissues.

Within selected genes, 34 polymorphisms were selected based on published reports and/or their locations in the genes, based on their likelihood to have functional consequences (i.e., located in the promoters, exons, or near exon/intron boundaries), or if considered tag-SNPs as surrogates for the linkage disequilibrium blocks surrounding the candidate gene. We used information from the NCBI dbSNP (<http://www.ncbi.nlm.gov/SNP/>) and the HapMap Project (<http://www.hapmap.org>) databases. Details of selected genes and polymorphisms are presented in Table 1.

Genotyping—Genomic DNA was extracted from saliva using established protocols. Genotypes were generated blindly to clinical diagnosis status. Reactions were carried out using Taqman chemistry (Ranade et al., 2001) in 5- μ L volumes in an ABI PRISM Sequence Detection System 7900 (Applied Biosystems, Foster City, CA). Assays and reagents were supplied by Applied Biosystems. The results were analyzed using SDS software version 1.7 (Applied Biosystems, Foster City, CA). In order to ensure quality control of genotyping reactions, we used a non-template control (using water instead of DNA) as a negative control and a sample of DNA of previously known genotype as a positive control. Genotype reactions were repeated twice when needed.

Statistical Analyses

Power analyses—Power calculations were performed using Genetic Power Calculator (Purcell et al. 2003) and indicate that the study sample size (~200 cases and ~500 controls) will provide approximately 84% statistical power to detect an association with an alpha of 0.05, if the markers selected are in LD with the causal factor ($D' = 0.8$) and their frequencies are around 20%.

Association analyses—Allele frequencies and Hardy-Weinberg equilibrium were calculated using PLINK software version 1.06 (Purcell et al. 2007). Analyses were performed comparing cases with chronic periodontitis and controls. We used Bonferroni correction as implemented in PLINK to adjust for multiple testing. We considered a significant level of 0.05 divided by the number of independent tests (32) to give a corrected P -value ($\alpha=0.002$).

Logistic regression analyses were performed under an additive model to assess the significance of the minor allele copy number on the association. Haplotype analyses were performed using 2-, 3-, and 4-SNP sliding windows.

Linkage disequilibrium analyses—Pairwise linkage disequilibrium (LD) values (D' and r^2) were calculated using Haploview version 4.1 (Barrett et al. 2005) for markers in *MMP3* for both populations. Measurements were obtained using genotypes of control samples as references.

Gene Expression Analysis

Biopsies of diseased ($n=128$) and healthy ($n=63$) gingival tissues were obtained from patients scheduled for treatment at the University of Ribeirão Preto School of Dentistry, and referred to periodontal surgery or surgical procedures due to either esthetics, orthodontic and prosthetic reasons (control tissues). Individuals signed a consent form approved by the local Institutional Review Board.

Total RNA was extracted from the tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and used for cDNA synthesis as previously described (Repeke et al. 2009).

Real Time-PCR was performed in triplicate reactions using 2.5ng of cDNA, specific primers and SYBR Green Master Mix (Invitrogen, Carlsbad, CA, USA) in a Mini Opticon system (BioRad, Hercules, CA, USA). Primers sequences were: for *MMP-3*, forward 5' ACCATCCTACAAATCTCGCGG, reverse 5' CATGGAGCTTGCTGCATTCTC; and for *TIMP-1*, forward 5' ACTGCAGGATGGACTCTTGCA, reverse 5' TTTCAGAGCCTTGAGGAGCT. Reaction conditions were 95°C (10'), and then 40 cycles of 94°C (1'), 56°C (1'), and 72°C (2'), followed by the standard denaturation curve.

Calculations for the relative levels of gene expression were determined from triplicate measurements of the target gene, with normalization to β -actin in the sample, using the cycle threshold (Ct) method and the $2^{\Delta\Delta Ct}$ equation, as previously described (Repeke et al. 2009). Analyses were performed comparing chronic periodontitis and control groups, and also comparing active or inactive chronic periodontitis lesions, as previously described (Menezes et al., 2008). A $p<0.05$ was considered statistically significant using Mann-Whitney test.

RESULTS

Genotype call rates were >99% for all investigated SNPs. Only the distributions of the genotypes derived from the Brazilian population for *MMP3* SNP rs522616 were not

compatible with those expected from Hardy-Weinberg equilibrium ($P < 0.05$), and this SNP was excluded from further analysis. For all other SNPs, no evidence of deviation from Hardy-Weinberg equilibrium was found.

The results show strong association of a SNP in *TIMP1* (rs5906435) with chronic periodontitis in the Brazilian population ($P = 0.0004$), whereas several SNPs in *MMP3* showed association in the US population, with the most significant associations observed for SNPs rs679620 ($P = 0.0003$) and rs650108 ($P = 0.002$). Although not enough to reach formal significance required by the strict Bonferroni correction, *MMP3* SNP rs639752 also showed a trend for association in the Brazilian ($P = 0.005$) and in the US ($P = 0.01$) populations, and additional associations were observed with *MMP3* rs522616 ($P = 0.004$) and *MMP2* rs243847 in the US ($P = 0.003$) (Table 2). Logistic regression analyses confirmed the observed associations for *MMP3* rs650108 and rs522616 in the US dataset ($P = 0.003$ and $P = 0.005$, respectively) (data not shown).

Haplotype analysis showed the most interesting results when viewing 2-, 3-, and 4-SNP sliding windows across the *MMP3* gene, namely in the area of SNPs rs679620 and rs650108 for the US population, and in the area of SNP rs639752 for the Brazilian population. In the US dataset, we observed significant associations for the combined haplotypes of SNPs rs650108-rs520540 (AG) ($P = 0.001$), and rs679620-rs522616 (CA) ($P = 0.002$). Additionally, the combination of alleles in rs639752-rs650108-rs520540 (AAG), rs650108-rs520540-rs679620 (AGC), and rs639752-rs650108-rs520540-rs679620 (AAGC) were also positively associated with chronic periodontitis in the US population ($P = 0.002$) (Table 3). Although no single individual *MMP3* SNP showed significant association with chronic periodontitis in the Brazilian population, we found strong association of SNP haplotypes rs639752-rs650108 (GG) ($P = 0.0001$), and rs639753-rs650108-rs520540-rs679620 (GGAG) ($P = 0.002$) (Table 3).

Figure 1 shows the results of linkage disequilibrium analyses for *MMP3* markers both populations. We found evidence of linkage disequilibrium for all *MMP3* markers in the US population, and in all but one SNP (rs639752) in the Brazilian population. This trend may justify the number of P-values lower than 0.05 found in the US dataset (Figure 1). When markers are in linkage disequilibrium, it is likely that they are overtransmitted together from parent to offspring and may be contributing to an association signal in combination with each other rather than individually. In such cases, it may be that any one of the SNPs in linkage disequilibrium represents a true causal association.

Figures 2 and 3 show the results of *MMP3* and *TIMP1* gene expression analyses in chronic periodontitis and healthy periodontal tissues. Overall, we observed an overexpression of both *MMP3* and *TIMP1* transcript levels in the diseased periodontal tissues when compared to healthy periodontal tissues (Figure 2A and 2B). When comparing among active or inactive lesions, the results show significantly higher *TIMP1* mRNA levels in inactive lesions, whereas the *MMP3/TIMP1* expression ratio appears higher in active lesions (Figure 2D and E). No significant differences were observed for the expression of *MMP3* in active or inactive lesions (Figure 2C). Upon correlation with disease clinical parameters (probing depth, bleeding on probing, and clinical attachment level), a positive association ($p = 0.0077$, $r^2 = 0.6526$) was found between the *MMP3/TIMP1* expression ratio and the observed clinical attachment levels (Figure 3).

DISCUSSION

Periodontitis is an infectious disease that is initially caused by oral bacteria in the gingival sulcus and affects the supporting connective tissues of the tooth. It is a complex

multifactorial condition where determining the importance of different risk factors is difficult (Hart & Kornman 1997). Several studies have suggested that a significant amount of variation between patients is the result of genetic predisposition, and the carriage of certain alleles appears to be related to the incidence and severity of periodontal disease (Vijayalakshmi et al. 2010).

Matrix metalloproteinases are a family of metal-dependent proteolytic enzymes that degrade extracellular matrix and basement membranes. They have been identified in inflamed periodontal tissues and may be related to the destruction of periodontal tissues (Hannas et al. 2007). Genetic variations in MMP genes can influence the level of their transcription and protein production. This may affect the connective tissue degradation properties of the protein and lead to the development of periodontitis. In the last years, reports of polymorphisms in several genes including interleukins (*IL1*, *IL6*, *IL8*, *IL10*, *IL17*) (Takahashi et al. 2005; Drozdik et al. 2006; Muller et al. 2007; Sumer et al. 2007; Andia et al. 2010; Nibali et al. 2010; Scarel-Caminaga et al. 2010), *TNFA* (Soga et al. 2003), and a few MMP genes (*MMP1*, *MMP3*, *MMP8*, *MMP9*) (de Souza et al. 2003; Holla et al. 2004; Astolfi et al. 2006; Repeke et al. 2009; Chou et al. 2011) have rendered important clues to the pathogenesis of periodontal disease. However, most genetic association studies on periodontitis have usually included an individual population and a single or a few polymorphisms within a given gene, thus not capturing the complete genetic information of the region of interest (Schäfer et al. 2011).

In the present study, we investigated 34 *MMP* and *TIMP* gene variants for their association with chronic periodontitis in two distinct populations from Brazil and the US. With a total sample size of approximately 200 cases and 500 controls, the detected associations are within ~84% likelihood of representing a causal variant in these populations. Although the sample size may be considered a limitation of the study, the careful selection of biologically relevant candidate genes such as MMPs and TIMPs and the inclusion of numerous variants within these genes make this study a comprehensive association analyses of this gene family regarding the etiology of chronic periodontitis. It has been postulated that detecting associations with common genetic variants is the first most straightforward and cost-efficient step towards better understanding of the genetic architecture of a complex disease (Schäfer et al. 2011).

Overall, our results show that *TIMP1* and *MMP3* are likely players in the pathogenesis of chronic periodontitis in the studied populations. An intronic SNP in *TIMP1* (rs5906435) showed strong association with diseased status in the Brazilian population whereas in the US dataset, the strongest associations were seen with two *MMP3* SNPs (rs679620 and rs650108). Although not formally significant under Bonferroni correction, additional borderline associations were observed for *MMP3* in both populations, and for *MMP2* in the US. The observed association with SNP rs679620 in the US dataset warrants additional studies as it represents a missense mutation (+700 G/A) in exon 2 of the *MMP3* gene, resulting in a conservative change of a lysine for a glutamine residue that may be involved in splicing regulation. *In silico* analysis of SNP functionality revealed that the minor allele A abolishes two exonic splicing enhancer motifs that can affect the final form of the encoded protein. Of note, several haplotypes including the individually associated SNPs in *MMP3* were also significantly associated with the disease. This suggests that the area nearby SNP rs679620 should be the focus of further study.

Our association findings with *MMP3* are supported by two additional case-control studies on chronic periodontitis (Astolfi et al. 2006; Loo et al. 2011). The first study, by Astolfi et al. (2006), consisted of 114 individuals with chronic periodontitis and 109 controls from Brazil, and found association of a promoter polymorphism in *MMP3* (rs3025058;-1171

5A6A) with the disease. Moreover, the authors reported that individuals presenting the 5A5A genotype were 3 times more likely to develop periodontitis (Astolfi et al. 2006). The second study, performed by Loo et al. (2011), comprised 280 chronic periodontitis cases and 250 controls from a Chinese population, and showed an association of the 5A5A genotype contributing to a two-fold higher risk of developing the disease (Loo et al. 2011). The -1171 5A6A polymorphism is a well-characterized insertion/deletion variant in its promoter characterized by the presence of either five or six adenine residues. The 5A allele has been shown to result in higher *MMP3* expression and enzyme activity and thereby increased extracellular matrix breakdown because of disruption of a binding site for a nuclear factor kappa B, which acts as a transcriptional repressor (Borghaei et al. 2004). In the present study, a nonsignificant trend for association was detected with the -1171 5A6A variant in the US dataset, where controls showed a higher frequency of the 6A allele, supposedly a protective variant.

Intronic variants in *TIMP1* also showed strong association with chronic periodontitis in the Brazilian population, and, to our best knowledge, this is the first report of association of *TIMP1* with chronic periodontitis. Tissue inhibitors of metalloproteinases (TIMPs) are naturally occurring inhibitors of MMPs, and have the ability to inhibit the catalytic activity of MMPs. In addition to an inhibitory role, TIMPs can also take part in the activation of MMPs or have biological activities that are independent of metalloproteinases and these include effects on cell growth and differentiation, cell migration, anti-angiogenesis, and anti- and pro-apoptosis. These activities may partially arise from MMP inhibition, as the regulation of extracellular matrix catabolism can influence cellular behavior, however many of them have been shown to be independent of MMP inhibition, because synthetic MMP inhibitors or TIMP mutants that do not inhibit MMPs can exhibit a similar activity. In the latter case, TIMPs interact directly with specific cell surface receptors to induce cellular responses (Brew and Nagase 2010). TIMP-1 enzyme levels are significantly higher in the plasma of individuals with chronic periodontitis (Soder et al. 2009). Moreover, it has been reported that *TIMP1* is also strong inhibitor of *MMP3* (Brew and Nagase 2010). Therefore, while the observed associations of *MMP3* and *TIMP1* chronic periodontitis in the Brazilian population might be related, additional studies in other populations must be performed in order to evaluate a synergistic or individual contribution of these genetic variants to disease susceptibility.

It is well accepted that the balance between MMPs and TIMPs plays a vital role in maintaining the integrity of healthy tissues, whereas disturbances in such balance is found in various pathologic conditions, including rheumatoid arthritis, periodontitis and cancer (Lambert et al. 2004). A previous study demonstrated that *MMP3* gene transcript levels were elevated in individuals with chronic periodontitis when compared to periodontally healthy subjects, although the difference was not statistically significant (Kubota et al. 2008). In contrast, the results of our expression analysis showed significantly increased levels of *MMP3* and *TIMP1* mRNA levels in the chronic periodontitis tissues when compared to healthy periodontal tissues. This suggests a likely attempt of the host environment to contain and perhaps outbalance the increased MMP (and maybe *MMP3* specifically) levels to minimize tissue damage. Moreover, when the chronic periodontitis tissues were categorized as active or inactive lesions based on a *RANKL/OPG* expression ratio (as previously described in Menezes et al. 2008), the observed levels of *TIMP1* were significantly higher in inactive lesions, thus reinforcing a potentially protective event. We observed a significantly higher *MMP3/TIMP1* expression ratio in the active lesions and a positive correlation with the individual's clinical attachment level, a specific clinical parameter indicative of the cumulative tissue loss due to periodontitis evolution.

Smoking is a known risk factor for periodontitis, and the association between smoking and periodontitis has been well documented (Hart & Kornman 1997). One of the limitations of the present study is the lack of analyses of smoking status in combination with risk genotypes. It has been reported that a genetic association with periodontitis was evident only in non-smokers (Kornman et al. 1997), which confirms the importance of smoking as a confounder in periodontal epidemiological studies. Hence, smoking should be considered additional threshold for disease predisposition, acting as a modifier to an individual's susceptibility to develop periodontitis, which may have a cumulative/chronic effect of this genotype over time.

The results of this study confirm previous findings of association of genetic variations in *MMP3* with chronic periodontitis, and report a novel association with *TIMP1* variants. Increased transcript levels of these enzymes were observed in periodontally compromised tissues when compared to healthy periodontal tissues, thus raising a question if these gene variants may contribute to imbalances in enzyme production and have an effect on the tissue breakdown associated with the disease. The different variants associated with distinct population groups, and the observed differences in minor allele frequencies of the investigated polymorphisms justify the individual associations in the particular populations, and also highlight the importance of allelic heterogeneity when interpreting results of genetic studies. Common alleles are more likely to be found globally. If so, it is expected that a causal association between a candidate SNP and trait outcome should be reproducible in many ethnically diverse populations. However, whereas pan-ethnic replicability provides support for a causal relationship, its absence does not necessarily negate it. It is possible that more than one variant might contribute to a particular condition and these variants may be different across populations. In addition, it is well known that the same mutation can cause a major disease phenotype in one strain of mouse but no phenotype in a genetically distinct strain. Thus, background factors (genetic and otherwise) differentiating populations can modify the expression of a gene and lead to different levels of association. For example, this seems to be the case for the *ApoE* gene and Alzheimer's disease, where the association exists pan-ethnically but is strongest in Caucasians and Asians, and weaker in Hispanics and African Americans (Farrer et al., 1997). Further, it is likely that most genuine genetic associations represent modest effects with odds ratios of 1.1–1.5 (i.e. a 10–50% relative increase in the likelihood of getting a disease). Any one polymorphism usually explains only 1–8% of the overall disease risk in a population. This might sound quite small, but the additive effect of several such risk factors could make up the 20–70% of the overall disease risk that is attributed to genetic factors in most common diseases, including periodontal disease (Ioannidis, 2003). Hence, whilst the specific mechanisms through which *MMP3* and *TIMP1* genes may contribute to an individual's disease susceptibility to chronic periodontitis are yet to be determined, these findings implicate the turnover of the extracellular matrix as an important and variable component of periodontal disease pathogenesis. Efforts to understand this process could form the basis for developing effective treatments for chronic periodontitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Armitage GC. Periodontal diagnoses and classification of periodontal diseases. *Periodontology* 2000. 2004; 34:9–21. [PubMed: 14717852]
- Andia DC, de Oliveira NF, Letra A, Nociti FH Júnior, Line SRP, de Souza AP. IL8 Gene Promoter Polymorphism (rs4073) may Contribute to Chronic Periodontitis. *Journal of Periodontology*. 2011; 82:893–899. [PubMed: 21091348]
- Astolfi CM, Shinohara AL, da Silva RA, Santos MC, Line SRP, de Souza AP. Genetic polymorphisms in the MMP-1 and MMP-3 genes may contribute to chronic periodontitis in a Brazilian population. *Journal of Clinical Periodontology*. 2006; 33:699–703. [PubMed: 16899023]
- Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 2005; 21:263–265. [PubMed: 15297300]
- Borghaei RC, Rawlings PL Jr, Javadi M, Woloshin J. NF-kappaB binds to a polymorphic repressor element in the MMP-3 promoter. *Biochemical and Biophysical Research Communications*. 2004; 316:182–188. [PubMed: 15003528]
- Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. *Biochimica et Biophysica Acta*. 2010; 1803:55–71. [PubMed: 20080133]
- Chou YH, Ho YP, Lin YC, Hu KF, Yang YH, Ho KY, Wu YM, His E, Tsai CC. MMP-8 -799 C>T genetic polymorphism is associated with the susceptibility to chronic and aggressive periodontitis in Taiwanese. *Journal of Clinical Periodontology*. 2011; 38:1078–1084. [PubMed: 22092455]
- de Souza AP, Trevilatto PC, Scarel-Caminaga RM, Brito RB, Line SR. MMP-1 promoter polymorphism: association with chronic periodontitis severity in a Brazilian population. *Journal of Clinical Periodontology*. 2003; 30:154–158. [PubMed: 12622858]
- Drozdzik A, Kurzawski M, Safronow K, Banach J. Polymorphism in interleukin-1 beta gene and the risk of periodontitis in a Polish population. *Advances in Medical Sciences*. 2006; 51 (Suppl 1):13–17. [PubMed: 17458053]
- Ejeil AL, Igondjo-Tchen S, Ghomrasseni S, Pellat B, Godeau G, Gogly B. Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva. *Journal of Periodontology*. 2003; 74:188–195. [PubMed: 12666707]
- Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance MA, Risch N, van Duijn CM. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *Journal of the American Medical Association*. 1997; 278:1349–1356. [PubMed: 9343467]
- Garlet GP, Martins W Jr, Fonseca BA, Ferreira BR, Silva JS. Matrix metalloproteinases, their physiological inhibitors and osteoclast factors are differentially regulated by the cytokine profile in human periodontal disease. *Journal of Clinical Periodontology*. 2004; 31:671–679. [PubMed: 15257746]
- Hannas AR, Pereira JC, Granjeiro JM, Tjaderhane L. The role of matrix metalloproteinases in the oral environment. *Acta Odontol Scand*. 2007; 65:1–13. [PubMed: 17354089]
- Hart TC, Kornman KS. Genetic factors in the pathogenesis of periodontitis. *Periodontology* 2000. 1997; 14:202–215. [PubMed: 9567972]
- Hollá LI, Jurajda M, Fassmann A, Dvorakova N, Znojil V, Vacha J. Genetic variations in the matrix metalloproteinase-1 promoter and risk of susceptibility and/or severity of chronic periodontitis in the Czech population. *Journal of Clinical Periodontology*. 2004; 31:685–690. [PubMed: 15257748]
- Ingman T, Tervahartiala T, Ding Y, Tschesche H, Haerian A, Kinane DF, Konttinen Y, Sorsa T. Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva of periodontitis patients. *Journal of Clinical Periodontology*. 1996; 23:1127–1132. [PubMed: 8997658]

- Ioannidis JP. Genetic associations: false or true? *TRENDS in Molecular Medicine*. 2003; 9:135–138. [PubMed: 12727138]
- Kornman KS, Crane A, Wang HY, di Giovine FS, Newman MG, Pirk FW, Wilson TG Jr, Higginbottom FL, Duff GW. The interleukin-1 genotype as a severity factor in adult periodontal disease. *Journal of Clinical Periodontology*. 1997; 24:72–77. [PubMed: 9049801]
- Kubota T, Itagaki M, Hoshino C, Nagata M, Morozumi T, Kobayashi T, Takagi R, Yoshie H. Altered gene expression levels of matrix metalloproteinases and their inhibitors in periodontitis-affected gingival tissue. *Journal of Periodontology*. 2008; 79:166–173. [PubMed: 18166107]
- Lambert E, Dassé E, Haye B, Petitfrère E. TIMPs as multifacial proteins. *Critical Reviews in Oncology/Hematology*. 2004; 49:187–198. [PubMed: 15036259]
- Letra A, Silva RA, Menezes R, Astolfi CM, Shinohara A, de Souza AP, Granjeiro JM. MMP gene polymorphisms as contributors for cleft lip/palate: association with MMP3 but not MMP1. *Archives of Oral Biology*. 2007; 52:954–960. [PubMed: 17537400]
- Loo WT, Wang M, Jin LJ, Cheung MN, Li GR. Association of matrix metalloproteinase (MMP-1, MMP-3 and MMP-9) and cyclooxygenase-2 gene polymorphisms and their proteins with chronic periodontitis. *Archives of Oral Biology*. 2011 Apr 8. [Epub ahead of print].
- Makela M, Salo T, Uitto VJ, Larjava H. Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. *Journal of Dental Research*. 1994; 73:1397–1406. [PubMed: 8083435]
- Menezes R, Garlet TP, Letra A, Bramante CM, Campanelli AP, Figueira R, de C, Sogayar MC, Granjeiro JM, Garlet GP. Differential patterns of receptor activator of nuclear factor kappa B ligand/osteoprotegerin expression in human periapical granulomas: possible association with progressive or stable nature of the lesions. *Journal of Endodontics*. 2008; 34:932–938. [PubMed: 18634923]
- Muller HP, Barrieshi-Nusair KM. A combination of alleles 2 of interleukin (IL)-1A (–889) and IL-1B (+3954) is associated with lower gingival bleeding tendency in plaque-induced gingivitis in young adults of Arabic heritage. *Clinical Oral Investigation*. 2007; 11:297–302.
- Nibali L, Donos N, Farrell S, Ready D, Pratten J, Tu YK, D’Aiuto F. Association between interleukin-6 -174 polymorphism and *Aggregatibacter actinomycetemcomitans* in chronic periodontitis. *Journal of Periodontology*. 2010; 81:1814–1819. [PubMed: 20681812]
- Nunn ME. Understanding the etiology of periodontitis: an overview of periodontal risk factors. *Periodontology 2000*. 2003; 32:11–23. [PubMed: 12756030]
- Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. PLINK: a tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics*. 2007; 81:559–575. [PubMed: 17701901]
- Purcell S, Cherny SS, Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics*. 2003; 19(1):149–150. [PubMed: 12499305]
- Rai B, Kharb S, Jain R, Anand SC. Biomarkers of periodontitis in oral fluids. *Journal of Oral Science*. 2008; 50:53–56. [PubMed: 18403884]
- Ranade K, Chang MS, Ting CT, Pei D, Hsiao CF, Olivier M. High-throughput genotyping with single nucleotide polymorphisms. *Genome Research*. 2001; 11:1262–1268. [PubMed: 11435409]
- Repeke CE, Trombone AP, Ferreira SB Jr, Cardoso CR, Silveira EM, Martins W Jr, Trevilatto PC, Silva JS, Campanelli AP, Garlet GP. Strong and persistent microbial and inflammatory stimuli overcome the genetic predisposition to higher matrix metalloproteinase-1 (MMP-1) expression: a mechanistic explanation for the lack of association of MMP1-1607 single-nucleotide polymorphism genotypes with MMP-1 expression in chronic periodontitis lesions. *Journal of Clinical Periodontology*. 2009; 36:726–738. [PubMed: 19659894]
- Scarel-Caminaga RM, Kim YJ, Viana AC, Curtis KM, Corbi SC, Sogumo PM, Orrico SR, Cirelli JA. Haplotypes in the Interleukin 8 Gene and Their Association with Chronic Periodontitis Susceptibility. *Biochemical Genetics*. 2011; 49:292–302. [PubMed: 21188496]
- Schafer AS, Jepsen S, Loos BG. Periodontal genetics: a decade of genetic association studies mandates better study designs. *Journal of Clinical Periodontology*. 2011; 38:103–107. [PubMed: 21158895]
- Söder PO, Meurman JH, Jogestrand T, Nowak J, Söder B. Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1 in blood as markers for early atherosclerosis in subjects

- with chronic periodontitis. *Journal of Periodontal Research*. 2009; 44:452–458. [PubMed: 18973519]
- Soga Y, Nishimura F, Ohyama H, Maeda H, Takashiba S, Murayama Y. Tumor necrosis factor-alpha gene (TNF-alpha) -1031/-863, -857 single-nucleotide polymorphisms (SNPs) are associated with severe adult periodontitis in Japanese. *Journal of Clinical Periodontology*. 2003; 30:524–531. [PubMed: 12795791]
- Sumer AP, Kara N, Keles GC, Gunes S, Koprulu H, Bagci H. Association of interleukin-10 gene polymorphisms with severe generalized chronic periodontitis. *Journal of Periodontology*. 2007; 78:493–497. [PubMed: 17335372]
- Takahashi K, Azuma T, Motohira H, Kinane DF, Kitetsu S. The potential role of interleukin-17 in the immunopathology of periodontal disease. *Journal of Clinical Periodontology*. 2005; 32:369–374. [PubMed: 15811054]
- Teng YT, Sodek J, McCulloch CA. Gingival crevicular fluid gelatinase and its relationship to periodontal disease in human subjects. *Journal of Periodontal Research*. 1992; 27:544–552. [PubMed: 1403585]
- Viera A, Garrett JM. Understanding interobserver agreement: The Kappa Statistic. *Family Medicine*. 2005; 37:360–363. [PubMed: 15883903]
- Vijayalakshmi R, Geetha A, Ramakrishnan T, Emmadi P. Genetic polymorphisms in periodontal diseases: an overview. *Indian Journal of Dental Research*. 2010; 21:568–574. [PubMed: 21187627]

Clinical Relevance

An imbalance between matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) has been observed in association with tissue breakdown and periodontal disease. We hypothesize that genetic variation in these genes may be related to disease pathogenesis. We observed the association of *MMP3* and *TIMP1* with chronic periodontitis making them likely players in periodontitis susceptibility. Further, we observed higher expression of these genes in periodontal disease tissues implicating them in disease pathogenesis. Efforts to understand this process could form the basis for developing effective treatments for chronic periodontitis.

Figure 1.

Overview of the linkage disequilibrium plots of single nucleotide polymorphisms in *MMP3* in Brazilian (A) and US (B) populations. The boxes represent the marker pair relationship and it is plotted between two markers. The color of the boxes (intensity of the color) is based on the raw score for that marker pair. Squares are colored darker if the D' value is high (i.e., LD is strong), lighter shades indicate less LD. Empty dark squares mean $D'=1$ (i.e., complete LD between two SNPs). Note complete LD among markers in the US population.

Figure 2.

Results of real-time PCR analyses of *MMP3* and *TIMP1* mRNA expression levels in control subjects (C, n=63) and chronic periodontitis patients (CP, n=128). Results are presented as expression of each individual mRNAs (with normalization to beta-actin using the Ct method). A $p < 0.05$ was considered statistically significant (*). Note significantly higher *MMP3* (A) and *TIMP1* (B) expression in CP patients when compared to controls. Analyses were also performed based on the lesion activity profile (active or inactive). C. No significant differences were observed for *MMP3* expression among active or inactive lesions. D and E. *TIMP1* expression levels were markedly higher in inactive lesions (D), similarly to the *MMP3*/*TIMP1* ratio (E).

Figure 3.

Graphical representation of the correlation between the *MMP3/TIMP1* expression ratio and the clinical parameters of disease severity site: probing depth (A), mean probing depth (B), clinical attachment level (C) and bleeding on probing (D). Note positive correlation between *MMP3/TIMP1* expression ratio and clinical attachment level (C, $P=0.008$).

Table 1

Details of the genes and SNPs investigated in the studied populations.

Gene	dbSNP ID	Location (chromosome, base position)	SNP Function	Position in sequence (nucleotide change) ^a	Alleles ^b
<i>MMP2</i>	rs243865	chr. 16 54069297	5' near gene	-1306 C/T	C/T
	rs2285053	chr. 16 54069878	5' near gene	-735 C/T	C/T
	rs243847	chr. 16 54081499	Intron		T/C
	rs2287074	chr. 16 54084614	Coding synonymous	Thr460Thr (exon 9)	G/A
	rs9923304	chr. 16 54087802	Intron		C/T
	rs11639960	chr. 16 54090771	Intron		A/G
<i>MMP3</i>	rs243832	chr. 16 54096692	Intron		C/G
	rs639752	chr. 11 102212549	Intron		T/G
	rs650108	chr. 11 102213997	Intron		G/A
	rs520540	chr. 11 102214635	Coding synonymous	Ala362Ala (exon 8)	G/A
	rs679620	chr. 11 102218830	Missense	Lys45Glu (exon 2)	G/A
	rs522616	chr. 11 102220258	5' near gene	-709 A/G	A/G
<i>MMP7</i>	rs3025058	chr. 11 102221152	5' near gene	-1171 5A/6A	6A/5A
	rs11568818	chr. 11 101906871	5' near gene	-181 A/G	A/G
<i>MMP8</i>	rs3765620	chr. 11 102100702	Missense	Thr32Ile	A/G
<i>MMP9</i>	rs3918253	chr. 20 44072918	Intron	49 5' exon 4	C/T
	rs17576	chr. 20 44073632	Missense	Gln279Arg	A/G
	rs17577	chr. 20 44076518	Missense	Gln668Arg (exon 12)	G/A
	rs13925	chr. 20 44078372	Condng synonymous	Val694Val	G/A
<i>MMP10</i>	rs17293607	chr. 11 102155599	Missense	Gly65Arg (exon 2)	C/T
<i>MMP13</i>	rs2252070	chr. 11 102331749	5' near gene	-77 A/G	G/A
<i>MMP14</i>	rs1042704	chr. 14 22382434	Missense	Asn273Asp	A/G
<i>MMP15</i>	rs2304488	chr. 16 58074210	Intron		A/G
<i>MMP16</i>	rs2616490	chr. 8 89150846	Missense	Ile415Val	A/G

Gene	dbSNP ID	Location (chromosome, base position)	SNP Function	Position in sequence (nucleotide change) ^a	Alleles ^b
	rs7828497	chr. 8 89408837	5' near gene		T/G
<i>MMP25</i>	rs2360167	chr. 16 3036160	5' near gene		G/T
<i>MMP27</i>	rs2509010	chr. 11 102067910	Missense	Asp447Asn	C/T
	rs12099177	chr. 11 102081592	Missense	Arg22Trp	G/A
	rs11225389	chr. 11 102081678	5' untranslated region		C/A
<i>TIMP1</i>	rs6520279	chr. X 47204350	Intron		C/T
	rs5906435	chr. X 47204664	Intron		C/T
	rs1062849	chr. X 47330943	Missense	Ser178Phe	C/T
<i>TIMP2</i>	rs8179096	chr. 17 74433244	5' near gene	-180 C/T	C/T
	rs9894526	chr. 17 74438681	5' near gene		C/T

^a According to NCBI Reference Assembly.

^b Ancestral allele listed first according to NCBI Reference Assembly.

^c Ancestral allele not available.

Table 2

Results of allelic association analyses for SNPs in MMP and TIMP genes with chronic periodontitis in Brazil and US populations.

Gene	dbSNP ID	Alleles ^a (1/2)	MAF ^b Brazil	MAF ^b US	Brazil (n= 401)				P-value ^c	US (n= 274)				P-Value ^c
					Cases (n=99)		Controls (n=302)			Cases (n=70)		Controls (n=204)		
					11	22	11	22		11	22	11	22	
MMP2	rs243865	C/T	0.21 (T)	0.19 (T)	143	37	481	113	0.6487	103	31	318	84	0.5845
	rs2285053	C/T	0.11 (T)	0.13 (T)	175	23	504	82	0.3958	117	11	346	44	0.3916
	rs243847	T/C	0.43 (C)	0.36 (C)	114	68	376	276	0.2286	73	63	227	167	0.003
	rs2287074	G/A	0.39 (A)	0.41 (A)	113	71	353	249	0.5024	81	55	247	151	0.6048
	rs9923304	C/T	0.38 (T)	0.41 (T)	102	76	357	241	0.5681	79	55	249	147	0.4188
	rs11639960	A/G	0.32 (G)	0.35 (G)	130	64	388	212	0.5512	96	44	275	127	1
MMP3	rs243832	C/G	0.48 (C)	0.49 (G)	91	83	263	261	0.6298	62	60	178	198	0.5039
	rs639752	T/G	0.48 (G)	0.49 (T)	72	108	312	290	0.005	83	53	195	201	0.0175
	rs650108	G/A	0.26 (A)	0.35 (G)	114	72	387	203	0.2847	50	88	91	307	0.002
	rs520540	G/A	0.49 (A)	0.43 (A)	99	65	331	261	0.3081	83	57	189	203	0.0244
	rs679620	G/A	0.48 (A)	0.41 (A)	108	70	350	252	0.5462	77	35	195	199	0.0003
	rs522616 ^d	A/G	0.20 (A)	0.49 (A)	----	---	----	----	----	35	85	59	289	0.004
MMP7	rs3025058	5A/6A	0.50 (5A)	0.39 (5A)	117	69	356	242	0.4117	78	56	193	211	0.0362
MMP8	rs11568818	A/G	0.49 (G)	0.43 (G)	109	89	319	239	0.6054	71	51	160	172	0.06
MMP9	rs3765620	A/G	0.40 (G)	0.38 (G)	114	66	342	218	0.5872	84	54	233	122	0.3215
	rs3918253	C/T	0.45 (C)	0.49 (C)	93	85	292	306	0.4233	61	79	183	219	0.6894
	rs17576	A/G	0.37 (G)	0.34 (G)	113	67	378	210	0.7123	90	48	254	150	0.6211
	rs17577	G/A	0.15 (A)	0.36 (A)	148	34	524	76	0.0409	120	20	341	61	0.7995
MMP10	rs13925	G/A	0.18 (A)	0.13 (A)	148	33	474	70	0.0733	92	20	278	62	1
MMP13	rs17293607	C/T	0.12 (T)	0.49 (T)	166	32	496	84	0.5668	121	17	351	47	0.8735
MMP14	rs2252070	A/G	0.33 (G)	0.33 (G)	94	56	387	193	0.3501	102	38	263	143	0.08
MMP15	rs1042704	G/A	0.28 (A)	0.12 (G)	156	24	515	85	0.7773	91	33	251	97	0.7872
MMP15 ^e	rs2304488	A/G	0.21 (G)	0.07 (G)	141	41	452	126	0.8360	----	----	----	----	----
MMP16	rs7828497	T/G	0.34 (G)	0.13 (G)	121	77	359	163	0.0514	93	45	264	136	0.7654

Gene	dbSNP ID	Alleles ^a (1/2)	MAF ^b Brazil	MAF ^b US	Brazil (n= 401)				P-value ^c	US (n= 274)				P-Value ^e
					Cases (n=99)		Controls (n=302)			Cases (n=70)		Controls (n=204)		
					11	22	11	22		11	22	11	22	
MMP25	rs2360167	G/T	0.21 (T)	0.35 (T)	156	28	460	140	0.0189	108	32	313	83	0.6382
	rs2509010	C/T	0.26 (T)	0.19 (T)	149	45	448	138	1	99	29	263	95	0.3874
MMP27	rs12099177	G/A	0.10 (G)	0.23 (G)	173	25	530	56	0.2198	13	117	36	342	0.8739
	rs11225389	C/A	0.23 (A)	0.21 (A)	139	29	482	112	0.6386	115	25	303	99	0.1005
TIMP1	rs6520279	T/C	0.47 (C)	0.31 (T)	80	88	331	251	0.0337	40	28	119	111	0.3035
	rs5906435	C/T	0.33 (T)	0.10 (T)	104	72	426	158	0.0004	44	22	155	73	0.8404
TIMP2	rs8179096	C/T	0.12 (T)	0.43 (T)	108	78	330	264	0.6364	98	8	321	49	0.1112
	rs9894526	C/T	0.43 (T)	0.18 (T)	108	78	330	266	0.5179	76	64	236	168	0.3944

^a Ancestral allele listed first according to NCBI Reference Assembly.

^b MAF, minor allele frequency in controls (minor allele in parenthesis).

^c Fisher exact test. Significant if P = 0.002, per Bonferroni correction (in bold).

^d *MMP3* SNP rs522616 excluded from analysis in Brazil due to deviation from Hardy-Weinberg equilibrium (P = 0.05).

^e *MMP15* SNPs rs2304488 was excluded from further analysis on the US population due to MAF <0.10.

Table 3

Results of haplotype analyses for polymorphisms in the *MMP3* gene in Brazil and US populations. Analyses were performed using 2-, 3-, and 4-SNP sliding windows (P-values in bold represent statistical significance per Bonferroni correction, P 0.002).

Brazil	Haplotype Window	rs639752	rs650108	rs520540	rs679620	
	2 SNP	0.0001 (GG)				
			0.0965			
					0.5216	
	3 SNP	0.09				
			0.05			
	4 SNP	0.002 (GGAG)				

US	Haplotype Window	rs639752	rs650108	rs520540	rs679620	rs522616	rs3025058
	2 SNP	0.004 (AA)					
			0.001 (AG)				
				0.05			
					0.002 (CA)		
						0.004 (A6A), 0.02 (G5A)	
	3 SNP	0.002 (AAG)					
			0.002 (AGC)				
				0.006 (GCA)			
					0.01 (CA6A)		
	4 SNP	0.002 (AAGC)					
			0.003 (AGCA)				
				0.01 (GCA6A)			