

The Association of -799C/T and -381A/G Matrix Metalloproteinase 8 (MMP-8) Gene Polymorphisms with Periodontitis and Active MMP-8 Levels in the Oral Cavity

SUMMARY

Background/Aim: To investigate the relationship of the -799C/T (*rs11225395*) and -381A/G (*rs1320632*) matrix metalloproteinase 8 (MMP-8) gene polymorphisms with periodontitis and the levels of active MMP-8 (aMMP-8) in an oral rinse. **Material and Methods:** Eighty subjects that had participated in a previous study contributed with an oral rinse sample collected before full-mouth periodontal clinical assessments. In addition, aMMP-8 levels in an oral rinse were quantified with a chairside point of care (PoC) test (PerioSafe®), and the accompanying digital reader (OraLyzer®). After DNA extraction, the samples were amplified with Polymerase Chain Reaction using specific primers. The amplified DNA samples were analyzed with a sequencing assay. **Results:** All of the patients were homozygous for the -381A/A genotype while 21.2% of the subjects were homozygous for the -799T/T genotype. No significant association was found between the -799T/T and periodontal disease, however, the presence of the specific genotype was significantly associated with the stage of periodontitis and the levels of aMMP-8 in the oral cavity. **Conclusions:** The results of the current study of 80 Greek subjects indicated that the presence of the *rs11225395* (-799 T/T) genotype may be correlated with the severity of the periodontal disease. In addition, patients homozygous for the T/T genotype had higher levels of aMMP-8.

Keywords: Periodontitis, Polymorphisms, MMP8

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Introduction

Periodontitis is a chronic inflammatory disease of microbial origin affecting the tooth-supporting apparatus^{1,2}. Periodontitis, if left untreated, eventually may lead to tooth loss. Therefore, periodontitis is considered a public health issue with financial and social aspects, leading to a reduced quality of life³. The microbial biofilm residing in gingival sulci and periodontal pockets is the main etiological factor of periodontitis, however, the disease is considered multifactorial implicating host defense mechanisms in its pathogenesis. Recently the World Workshop of Periodontology released the new periodontitis classification

including four different stages and three grades depending on several clinical and radiological parameters².

Enzymes called matrix metalloproteinases (MMPs) participate in the destruction of the extracellular matrix⁴. By regulating tissue remodeling, during wound healing, repair and inflammation, MMP-8 is responsible for collagen destruction. This enzyme also promotes the development of inflammation, and it is known that the enzymatic activity of MMP-8 directly influences the progression of periodontal disease by up or down-regulating the proteins and mediators involved in inflammation, neutrophil migration, and tissue healing^{5,6}. Increased MMP-8 expression in periodontal disease, particularly in the active form (aMMP-8), has been

reported in several studies^{5,7}. High levels of aMMP-8 expression in gingival crevicular fluid (GCF), serum, or saliva are strongly correlated with periodontitis and the severity of the periodontal disease^{4,6,8}. After nonsurgical treatment of periodontitis, levels of MMP-8 and its active form decrease to a non-disease level, and this reduction correlates to an improvement in clinical disease parameters⁹. Thus, MMP-8 is considered an important candidate biomarker for the diagnosis and prognosis of periodontitis progression, as well as the effects of different treatment modalities. For this reason, a chairside point of care (PoC) test has been developed to allow for real-time quantification of aMMP-8 levels in saliva (PerioSafe®, Dentognostics, GmbH, Jena, Germany), with the accompanying digital reader (OraLyzer®). The test has shown increased sensitivity and specificity in the early diagnosis of periodontal disease¹⁰.

The important role of MMP-8 regarding the pathogenetic processes of periodontal disease has prompted several researchers to investigate the possible association of MMP-8 gene polymorphisms with periodontitis. Several common single nucleotide polymorphisms (SNPs) have been identified in the MMP8 gene, which is located on chromosome 11. Two of them at the positions -799C/T (rs11225395), -381A/G (rs1320632) from the major transcription start site, have been described to have a possible correlation with periodontal disease¹¹⁻¹⁴. However, the results of those studies are inconclusive, as some of them agree on an association of those polymorphisms with periodontitis while others do not. In addition, only one study tried to evaluate a possible association in a Caucasian population and no similar studies exist in Greek population cohorts. Thus, the current study aimed to investigate the correlation of specific MMP8 gene polymorphisms with periodontitis and levels of aMMP-8, in a Greek population cohort.

Material and Methods

The study was conducted in the Department of Periodontology and Implant Biology, School of Dentistry, Aristotle University of Thessaloniki, Greece. A sample of patients from a previous was withdrawn study for this one¹⁵. In brief, participants were considered eligible if they met the following criteria: age >30 years, absence of systemic diseases or drugs known to affect periodontal tissues, absence of infectious diseases (hepatitis or HIV), absence of pregnancy or lactation, and absence of periodontal treatment or antibiotic use within the previous six months. Prior to taking part in the trial, each patient signed an informed consent form.

Participants were told to provide a 10-mL sample of unstimulated saliva in a sterile plastic centrifuge tube (Greiner Bio-one International GmbH). They were

instructed not to brush their teeth, eat or drink anything, or smoke for at least an hour before the saliva was collected. Until analysis, saliva samples were stored at -80°C. Additionally, a quantitative aMMP-8 point-of-care (PoC)/ chairside oral rinse test was used to measure the levels of active-matrix metalloproteinase-8 (aMMP8) in accordance with the manufacturer's instructions¹⁰. Following oral fluid collection, a full-mouth periodontal examination was carried out by one calibrated examiner using an automated probe (Florida Probe; Florida Probe Corporation, Gainesville, Florida, United States) (A.G.). Plaque, bleeding on probing (BoP), probing attachment level (PAL), and probing pocket depth (PPD) were all clinically measured during the periodontal examination. Patients were given a periodontal diagnosis based on the criteria of the latest classification of periodontal diseases². From an initial 150 subject sample, 80 DNA samples were selected for gene analysis depending on the quality of DNA after measurement of absorbance at spectrophotometer at 310nm.

DNA Extraction

DNA was extracted from saliva samples using the Quick-DNA Miniprep Plus Kit (Zymo Research; Irvine, California, United States), following the manufacturer's protocol. The extracted DNA was stored at -20°C until further analysis.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) for the MMP8 gene was performed using the following primers:

FW 5'-CTGTTGAAGGCCTAGAGCTGCTGCTCC-3'
 ,REV 5'-GATCTTCTCTTCAAACCTACTACCC-3'

The PCR amplification was carried out in a 25-μL reaction volume containing 2 μL of template DNA, 0.3 μM of each Primer (TIB MOLBIOL, Germany), 100 μM of each dNTP (dNTP Bundle, Jena Bioscience, Jena, Germany), 1X of KAPA Taq Buffer A 10X with 1.5 mM MgCl₂ (KAPATaq PCR kit, Kapa Biosystems, Inc., Wilmington, Massachusetts, United States; Roche, Basel, Switzerland), 0.5U of KAPA Taq DNA Polymerase (Kapa Biosystems, Inc., Wilmington, Massachusetts, United States; Roche, Basel, Switzerland) and sterile ddH₂O. The PCR cycling conditions comprised an initial 2-minute heating step at 95°C, followed by 30 cycles of 30 seconds at 95°C, 1 minute at 62°C, and 1 minute at 72°C, and a final extension at 72°C for 5 minutes. Amplification products were run on 2% agarose electrophoresis gel stained with Midori green (NIPPON Genetics). The PCR fragments were purified using the Nucleospin Extract II kit by Macherey-Nagel (Duren, Germany), and the eluted amplicons were stored at -20 °C. Both PCR primers (forward and reverse) were used to sequence the amplified PCR products. The sequencing assay was carried out by

CEMIA (31 Str. Markrigianni, Larissa, Greece). Pairwise nucleotide alignments of the sequence obtained from the studied samples were performed with the sequence derived from the promoter region and 5' UTR of the MMP8 gene (AF059679) in order to search for the SNPs -799C/T (rs11225395) and -381A/G (rs1320632).

Statistical Analysis

Statistical analyses were performed with the IBM SPSS 25 Statistical Software Package (IBM Corporation, United States). Patient characteristics and their association with allele genotype were tested by Pearson's Chi-square test (asymptotic, two-sided). Differences regarding periodontal parameters assessed between sub-groups were tested with the Mann-Whitney *U*-test. The associations of allele and genotype frequencies in the patient and control groups were analyzed using Fisher's exact test of independence. The level of statistical significance was set at 0.05.

Results

The demographic characteristics of participants are presented in Table 1. The mean age of the participants was 51.4 (\pm 12.1) years old and male and female subjects were equally distributed in the investigated sample. Most patients were diagnosed with periodontitis. No patient received the diagnose stage IV periodontitis.

Table 1. Demographic Characteristics of The Patients

Sex	Male	Female
	52 (52.5%)	38 (47.5%)
Periodontal Status	Healthy	Periodontitis
	21 (26.3%)	59 (73.8%)
Smoking	Yes	No
	22 (27.5%)	58 (72.5%)

Genotype frequencies for rs11225395 (C/T) and rs1320632 (A/G) in the MMP8 gene and the distribution of alleles dependent on the periodontal condition are summarized in Table 2. All the participants were homozygous for the rs1320632 (A/A) polymorphism.

Table 2. Distribution of allele frequencies depending on the periodontal condition.

		Healthy	Periodontitis
rs11225395 (C/T)	CC	19 (30.2%)	44 (69.8%)
	TT	2 (11.8%)	15 (88.2%)
rs1320632 (A/G)	AA	21 (26.3%)	59 (73.8%)
	GG	0 (0%)	0 (0%)

The SNP rs11225395 (T/T) was found in 17/80 subjects. The comparison among periodontal condition

groups did not reach statistical significance ($p=0.108$, Chi-Square, Fisher's Exact Test, Table 3), although the specific polymorphism was numerally more frequent in patients with periodontitis. All of the patients examined were homozygous for the AA genotype in the case of rs1320632 SNP.

Table 3. Association of rs11225395 (C/T) genotype with periodontal disease and other parameters.

Variable	CC	TT	p-value
Periodontal Status ^a			
Health	19 (30.2%)	2 (11.8%)	0.106
Periodontitis	44 (69.8%)	15 (88.2%)	
Stage ^a			
Health	19 (30.2%)	2 (11.8%)	
Stage I	6 (9.5%)	2 (11.8%)	0.007*
Stage II	31 (49.2%)	5 (29.4%)	
Stage III	7 (11.1%)	8 (47.1%)	
Grading ^a			
Health	19 (30.2%)	2 (11.8%)	
Grade A	6 (9.5%)	2 (11.8%)	0.486
Grade B	34 (54%)	12 (70.6%)	
Grade C	4 (6.3%)	1 (5.9%)	
MMP8 Levels (ng/ml, Mean, SD) ^b	18.69 (16.15)	25.49 (21.92)	0.026*
Probing Depths (PD, Mean, SD mm) ^b	2.35	2.38	0.906
Clinical Attachment Levels (CAL, mean, SD, mm) ^b	2.43	2.45	0.878
Bleeding On Probing (BOP%) ^b	38.8%	38.3%	0.874

^aFischers Exact Test, ^bMan whithey Test, * Statistical Significance.

However, a significant association between the presence of the TT genotype and the stage of periodontal disease was evident ($p=0.007$, Pearson's Chi-Square Test). In addition, patients homozygous for the TT polymorphism exhibited statistically significant higher levels of aMMP-8 in their oral rinse ($p=0.026$, Man-Whitney Test). No association was found between the rs11225395 (C/T) polymorphism and Grading, Probing Depth, Probing attachment level, or Bleeding on Probing. However, a significant association between periodontitis and levels of aMMP8 was found ($p<0.001$, Pearson's Chi-Square, Fischer's Exact Test).

Discussion

The role of matrix metalloproteinase 8 (MMP-8) in the pathogenesis of periodontal disease has long been established. Considering the genetic factor as a potential co-driver in the initiation and progression of periodontal

disease several studies have tried to evaluate the role of various MMP-8 gene single nucleotide polymorphisms in periodontitis.

In the current study, the association of periodontitis with two specific gene polymorphisms of MMP-8 was investigated. The results revealed that in the specific study sample all the participants were homozygous for the rs1320632 (-381 A/G) genotype while no significant association was evident among rs11225395 (-799 C/T) polymorphism and periodontitis. This agrees with the findings from previous studies. Holla and coworkers compared the presence of -799C/T and 17C/G gene polymorphisms in 619 Caucasian patients with or without periodontal disease and although a significant relationship failed to be established, the haplotype -799T/+17G was more frequent in patients with periodontal disease¹⁵. Similarly, Chou *et al.*, in a study involving 361 Taiwanese patients with periodontal disease, 96 with aggressive periodontitis, and 106 healthy controls, failed to demonstrate any significant association between the -799C/T polymorphism and periodontitis¹¹. In contrast, a more recent study in the Indian population revealed a significant association of the -799C/T polymorphism with periodontitis in 357 Indian patients while *MMP8* +17G-C and *MMP3* -11715A- 6A mutant alleles were associated with decreased susceptibility to periodontitis¹⁴.

In the current study, a significant relationship between -799T/T polymorphism and the stage, thus the severity of periodontitis was observed, with patients diagnosed with stage III periodontitis being more likely to have the -799T/T genotype. Interestingly, another study in a Turkish population demonstrated a significant relationship between the investigated polymorphism with aggressive forms of periodontal disease indicating a potential association of the -799C/T polymorphism with the severity of periodontal disease¹². Most studies aimed to evaluate potential associations between periodontitis and MMP-8 polymorphisms do not involve a substantial number of subjects and only a few use large population samples. Thus, a potential association might remain hidden.

Matrix metalloproteinase-8 (MMP-8) in its active form is a potential biomarker for the diagnosis and prognosis of periodontal disease^{5,10}. Previous studies have shown a significant correlation between the levels of a-MMP-8 and the periodontal condition (i.e., Stage III)^{15,16}. This observation is corroborated by findings of the current study where the levels of aMMP-8 were correlated with the presence of periodontitis. In addition, patients homozygous for the rs11225395 (-799 T/T) polymorphism exhibited higher levels of a-MMP8 in an oral rinse. The only study assessing both the genomic profile of periodontal patients and the levels of MMP-8 in gingival crevicular fluid showed that carriers of the T allele of this specific polymorphism tended to have higher levels of MMP-8 compared to non-carriers, which

is in agreement with the present findings¹². However, an established correlation between periodontitis status and the specific (T/T) polymorphism could not be found. This is attributed to the fact that the sample size maybe was not sufficient to show a potential correlation. In addition, the existence of a T allele in the rs11225395 position of the MMP-8 gene might not be the only factor that results in the overexpression of MMP-8 protein in the gingival crevicular fluid. For example, the exact role of smoking has not been enlightened since the majority of the studied patients were nonsmokers.

Taken collectively, findings from previous and the present study suggest that the rs11225395 (-799 C/T) gene polymorphism appears to affect the pathogenetic mechanism of periodontal diseases¹⁷, however, larger-scale studies are required in order to amplify the results of the present study.

Conclusions

The results of the current study of 80 Greek subjects indicated that the presence of the rs11225395 (-799 T/T) genotype is correlated with the severity of the periodontal disease. Patients homozygous for the T/T genotype exhibited higher levels of active MMP-8 in the oral cavity.

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