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Matrix metalloproteinase-1 and -3 gene promoter polymorphisms in Japanese patients with periodontitis

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Abstract

Background/aims: Matrix metalloproteinase (MMP)-1 and MMP-3 have important roles in the connective tissue remodelling and destruction processes in periodontitis. MMP-1 1G/2G (−1607) and MMP-3 5A/6A (−1171) polymorphisms have been identified and appear to influence the transcription of the genes. The aim of this study was to investigate whether these gene promoter polymorphisms were associated with the susceptibility to periodontitis.

Material and Methods: Genomic DNA was obtained from 37 generalised aggressive, 205 slight-to-severe generalised chronic-periodontitis patients and 142 healthy subjects. All subjects were non-smoking Japanese. We genotyped by using TaqMan® PCR assay. The statistics were analysed by χ^2-test.

Results: We found no significant differences in genotype distributions, allele frequencies, carriage rates and haplotype frequencies in the MMP-1 and the MMP-3 gene promoter polymorphisms among all groups. The distributions of MMP-1 and MMP-3 genotypes in our study were different from those of previously reported in Caucasians or Brazilians, but consistent with previously reported in Japanese.

Conclusion: Our data did not support the hypothesis that MMP-1 and/or MMP-3 gene promoter polymorphisms influenced the susceptibility to periodontitis in Japanese patients, indicating MMP-1 and MMP-3 expressions were regulated by complex processes such as cytokine network in periodontal disease rather than gene polymorphisms.

Key words: genotypes; Japanese; matrix metalloproteinases; periodontitis

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Periodontal diseases are common in bacterially induced inflammatory conditions, which lead to the destruction of periodontal tissues. Matrix metalloproteinases (MMPs) are thought to be the most important enzymes in the connective tissue destruction, thus MMPs eventually degrade periodontal ligamental attachments and bone matrix proteins (Birkedal-Hansen 1993, Reynolds & Meikle 1997, Kinane 2000).

We have previously reported that the gene transcript levels for MMP-1 (interstitial collagenase), MMP-3 (stromelysin-1, activator of collagenases) and MMP-8 (neutrophil collagenase) were significantly elevated in periodontitis-affected gingival tissues than in healthy gingivae (Kubota et al. 1996). MMP-1 and MMP-3 protein levels were found to be increased in periodontitis-affected gingival specimens by morphometric and automated image analysis (Séguier et al. 2001). Moreover, the protein amounts and the activities of MMP-1 and MMP-3 in gingival crevicular fluid (GCF) and in gingival tissue were reported to be higher in periodontitis-affected sites than in healthy sites (Soell et al. 2002). These findings support our previous results suggesting that MMP-1 and MMP-3 are key players acting synergistically in the periodontal extracellular matrix degradation and remodelling.

Recently, functional single nucleotide polymorphisms (SNPs) of MMP-1 and MMP-3 has been described, and reports on the association between MMP gene promoter polymorphisms and various diseases have been accumulating such as in colorectal cancer

(Hinoda et al. 2002), carotid artery disease (Ghilardi et al. 2002, Lamblin et al. 2002) and acute myocardial infarction (Terashima et al. 1999).

The MMP-1 SNP at position –1607 is due to an insertion/deletion of a guanosine (G) (1G/2G). Two G allele is proven to create the core sequence motif (5′-GGA-3′) for the Ets family, resulting in higher transcriptional activities than the 1G allele in normal fibroblasts and in melanoma cells (Rutter et al. 1998).

The other MMP-3 SNP results from a variation in the length of a polymorphic track of adenosines (A) located at position –1171 (5A/6A). Five A allele is shown to have greater promoter activities compared with the 6A allele in fibroblasts and vascular smooth muscle cells (Ye et al. 1996).

In addition, both MMP-1 and MMP-3 genes are known to be adjacently localised in chromosome 11q22.3, and these two loci are in linkage disequilibrium and considered to act in cooperation (Hinoda et al. 2002).

All disease types of periodontitis, especially early onset (aggressive) periodontitis (AgP), have at least a genetic basis for susceptibility to periodontal microbial infection. Therefore, in this study, we investigated whether MMP-1 and/or MMP-3 SNPs are associated with susceptibility to periodontitis in a Japanese population. To determine the genotypes of these SNPs, we used a newly developed method, polymerase chain reaction (PCR), with fluorogenic probes (TaqMan® MGB probe), which is more reliable and faster than the standard PCR-restriction fragment length polymorphism (PCR-RFLP) assay (Schmitt et al. 2002).

Material and Methods

Subjects

Thirty-seven generalised AgP patients, 205 (56 severe, 84 moderate and 65 slight) chronic periodontitis (ChP) patients and 142 healthy (H) subjects were enrolled in this study. All subjects were non-smokers, Japanese, and none of them had a history or current manifestation of systemic disease. The patients had been referred to the Periodontal Clinic of Niigata University Dental Hospital, and were given a diagnosis of AgP, ChP or H. We used the criteria of Diehl et al. (1999) in diagnosing AgP. It was defined by the following clinical conditions: more than 5 mm attachment loss at more than one site on eight teeth, at least three of which were not first molars and incisors and the onset of the disease occurred less than 35 years of age. The ChP patients were further grouped according to the criteria of Kornman et al. (1997): a ChP patient with less than 15% bone loss was classified as slight, more than 33% bone loss was classified as severe, and from 15% to 33% bone loss was classified as moderate. The clinical profile of the patients is shown in Table 1. The H subjects (68 males, 74 females, mean age 25.4 ± 2.9 years), who showed neither attachment loss nor probing depth greater than 3 mm at more than one site, were matched by gender and age with AgP patients. The study was approved by the Institutional Review Board at Niigata University, Faculty of Dentistry, and written informed consent was obtained from all participants before inclusion in the study in accordance with the Helsinki declaration.

DNA extraction

Genomic DNA was obtained from peripheral blood containing whole blood cells by using a DNA extraction kit (Wako Pure Chemical Industries Inc., Osaka, Japan) according to the manufacturer's instructions. After the extraction, the DNA was diluted to a concentration of 70 ng/μl, and stored at −20°C until use.

Discrimination of genotype

We used the TaqMan® PCR assay based on the allelic discrimination using fluorogenic probes and 5′ nuclease described by Livak (1999). In brief, a fluorogenic probe, consisting of an oligonucleotide labelled with both a fluorescent reporter dye and a quencher dye, is included in a typical PCR. The amplification of the probe-specific product causes cleavage of the probe, generating an increase in reporter fluorescence. By using different reporter dyes (FAM or VIC), cleavage of allele-specific probes can be detected in a single PCR.

The specific forward/reverse PCR primers and TaqMan® MGB probes for MMP-1 and MMP-3 SNPs were designed with Primer Express Ver.1.5 Software (Applied Biosystems, Foster City, CA, USA), and custom synthesised by Applied Biosystems (Table 2).

Reaction mixture contained 1 μl of 70 ng/μl DNA solution, 2.25 μl of each primer (10 μM), 1 μl of each probe (5 μM), 12.5 μl of TaqMan® Universal Master Mix (Applied Biosystems) and 5 μl of distilled water in a final volume of 25 μl.

Negative controls (no template controls: NTCs) and positive controls whose genotypes were previously determined by direct sequencing were included in every assay.

The reaction mixtures were loaded into 96-well plates and placed in an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of PCR: denaturation at 92°C for 15 s, and one step annealing/extension for 1 min at 60°C (MMP-1) or 62°C (MMP-3).

Statistical analysis

χ^2-test was performed to examine differences in genotype distribution, allele frequency and carriage rate among three groups: AgP, ChP and H. Three ChP phenotypes according to the severity (slight, moderate or severe) of the disease were also analysed in the same way. A p-value of less than 0.02 was taken as statistically significant.

The haplotype analysis was carried out using the genotypic data at the two

Table 1. Characteristics of study populations

Diagnosis	*N*	Male	Female	Age (years)	PPD (mm)	CAL (mm)	BL (%)
AgP	37	9	28	31.4 ± 3.7	4.2 ± 0.9	4.9 ± 1.2	43.4 ± 13.4
ChP	205	76	129	52.8 ± 10.8	3.1 ± 1.1	3.7 ± 1.5	26.0 ± 15.0
severe	56	28	28	50.5 ± 11.4	4.3 ± 1.1	5.4 ± 1.5	46.3 ± 9.8
moderate	84	32	52	55.4 ± 11.0	3.0 ± 0.7	3.5 ± 0.7	23.7 ± 5.5
slight	65	16	49	51.6 ± 9.2	2.2 ± 0.4	2.4 ± 0.5	11.4 ± 2.3

AgP, aggressive periodontitis patients; ChP, chronic periodontitis patients; PPD, probing poket depth; CAL, clinical attachment level; BL, bone loss. Values represent mean ± SD.

Table 2. Sequences of PCR primers and TaqMan® MGB probes

	Forward primer	Reverse primer	TaqMan® MGB probe
MMP-1 (−1607)	TGCCACTTAGATG-AGGAAATTGTAGT	ACACTTTCCTCC-CCTTATGGATTC	1G:FAM: ATAATTAGAAAGAT-ATGACTTATC 2G:VIC: ATAATTAGAAAGGA-TATGACTTAT
MMP-3 (−1171)	ACATCACTGCCAC-CACTCTGTT	GGCACCTGGCC-TAAAGACATT	5A:FAM: AAGACATGGTTTTTC 6A:VIC: AGACATGGTTTTTTC

PCR, polymerase chain reaction; MMP, matrix metalloproteinase.

SNP sites by the maximum likelihood method using expectation–maximisation (EM) algorithm as described previously (Hinoda et al. 2002). χ^2-test was also used to examine differences in haplotype among all groups.

Results

In the present study, we have successfully genotyped the common MMP-1 SNP at nucleotide position −1607 (1G/2G) and the common MMP-3 SNP at nucleotide position −1171 (5A/6A) by using TaqMan® PCR assay in Japanese patients with periodontitis (Fig. 1a, b). As shown in Fig. 1, both MMP-1 and MMP-3 genotypes were separated by FAM or VIC fluorescence intensity. The genotypes were separated clearly in MMP-1 assay, but it was more difficult to separate 5A and 6A in MMP-3 assay. The weak separation was due to the limitation of the TaqMan® MGB probe hybridising to the adenosine-rich sequences (5A/6A). The MMP-3 genotyping was improved by the adjustment of the melting temperature (T_m) and the concentration of the probe. To confirm the correct genotyping, the samples that showed weak separation were further checked by direct sequencing.

The results of the genotype distribution, the allele frequencies and the allele carriage rates (frequency of individuals who carry at least one copy of polymorphic alleles) are summarised in Tables 3a and 3b. The genotype distributions for both MMP-1 and MMP-3 SNPs in H were in Hardy–Weinberg equilibrium (MMP-1: $\chi^2 = 0.50$, $p > 0.05$, MMP-3: $\chi^2 = 0.03$, $p > 0.05$).

As shown in Table 3a, MMP-1 2G/2G genotype distribution in AgP group seemed relatively low compared with ChP or H. Similarly, the 2G allele frequency and the 2G carriage rate in AgP were a bit lower than those of ChP or H; however, it failed to detect statistical differences. There was no MMP-3 5A/5A genotype in AgP, although the 5A carriage rate in AgP tended to be higher than those in H (carriage rate 45% in AgP versus 29.6% in H: $p = 0.059$) (Table 3a).

Table 3b shows the distribution among three ChP phenotypes: severe, moderate and slight. MMP-1 2G/2G genotype, 2G allele frequency and 2G carriage rate were a bit higher in severe ChP patients than slight group, however, there were no statistically significant differences ($p = 0.589$, 0.633, 0.767, respectively). On the other hand, MMP-3 5A/5A genotype, 5A allele frequency and 5A carriage rate were likely to be lower in severe group than in slight group. However, statistically significant differences could not be observed ($p = 0.240$, 0.188, 0.114, respectively).

The haplotype frequency was calculated by using the EH program (Table 4). The 1G-5A haplotype tended to be more numerous in AgP compared with ChP or H, although it did not reach statistically significant differences (AgP versus H: $p = 0.076$, AgP versus ChP: $p = 0.081$, ChP versus H: $p = 0.871$).

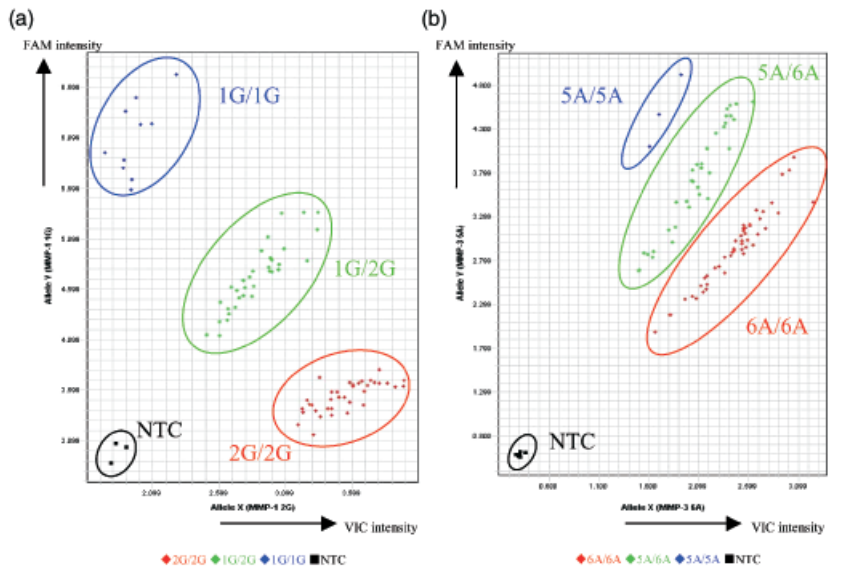

Fig. 1. Allelic discrimination with fluorogenic probes in the 5′ nuclease assay. (a) A person who carries MMP-1 2G/2G allele is represented as a red dot. Similarly, green dots represent 1G/2G and blue dots for 1G/1G carrier, respectively. Black dots are no template-negative controls (NTCs). *X*-axis: fluorescence intensity of VIC (2G); *Y*-axis: fluorescence intensity of FAM (1G). (b) A Person who carries MMP-3 6A/6A allele is represented as a red dot. Similarly, green dots represent 5A/6A and blue dots for 5A/5A carrier, respectively. Black dots are no NTCs. *X*-axis: fluorescence intensity of VIC (6A); *Y*-axis: fluorescence intensity of FAM (5A).

Discussion

We genotyped the linked two SNPs, MMP-1 (1G/2G, −1607) and MMP-3 (5A/6A, −1171), in AgP and ChP patients to investigate whether the sequence variation in MMP genes influenced susceptibility to periodontitis in Japanese patients. We could not see any significant association between these SNPs and the susceptibility to periodontal diseases.

We found that the genotype distribution in both MMP-1 and MMP-3 SNPs were consistent with the previous results in healthy Japanese populations (Kanamori et al. 1999, Terashima et al. 1999, Nishioka et al. 2000, Takahashi et al. 2001, Hinoda et al. 2002).

On the other hand, these genotype distributions in Japanese were found to

Table 3a. Distribution of MMP-1 and MMP-3 SNPs

Subjects	Frequency % (*n*)			*p*-Value (χ^2 test)		
	AgP (A)	ChP (C)	H	A versus C	A versus H	C versus H
MMP-1						
genotype						
2G/2G	35.1 (13)	47.3 (97)	41.5 (59)	0.334	0.720	0.558
1G/2G	46.0 (17)	40.0 (82)	43.7 (62)			
1G/1G	18.9 (7)	12.7 (26)	14.8 (21)			
total	(37)	(205)	(142)			
allele frequency						
2G	58.1 (43)	67.3 (276)	63.4 (180)	0.124	0.405	0.283
1G	41.9 (31)	32.7 (134)	36.6 (104)			
carriage rate						
2G+	81.1 (30)	87.3 (179)	85.2 (121)	0.309	0.538	0.573
2G−	18.9 (7)	12.7 (26)	14.8 (21)			
MMP-3						
genotype						
5A/5A	0.0 (0)	2.4 (5)	2.8 (4)	0.076	0.057	0.935
5A/6A	45.9 (17)	28.3 (58)	26.8 (38)			
6A/6A	54.1 (20)	69.3 (142)	70.4 (100)			
total	(37)	(205)	(142)			
allele frequency						
5A	23.0 (17)	16.6 (68)	16.2 (46)	0.184	0.173	0.892
6A	77.0 (57)	83.4 (342)	83.8 (238)			
carriage rate						
5A+	45.9 (17)	30.7 (63)	29.6 (42)	0.070	0.059	0.818
5A−	54.1 (20)	69.3 (142)	70.4 (100)			

Table 3b. Distribution of MMP-1 and MMP-3 SNPs among chronic periodontitis patients

	Frequency % (*n*)		
	severe	moderate	slight
MMP-1			
genotype			
1G/1G	12.5 (7)	14.3 (12)	10.8 (7)
1G/2G	33.9 (19)	41.7 (35)	43.1 (28)
2G/2G	53.6 (30)	44.0 (37)	46.1 (30)
total	(56)	(84)	(65)
allele frequency			
1G	29.5 (33)	35.1 (59)	32.3 (42)
2G	70.5 (79)	64.9 (109)	67.7 (88)
carriage rate			
2G+	87.5 (49)	85.7 (72)	89.2 (58)
2G−	12.5 (7)	14.3 (12)	10.8 (7)
MMP-3			
genotype			
5A/5A	3.6 (2)	1.2 (1)	3.1 (2)
5A/6A	21.4 (12)	27.4 (23)	35.4 (23)
6A/6A	75.0 (42)	71.4 (60)	61.5 (40)
total	(56)	(84)	(65)
allele frequency			
5A	14.3 (16)	14.9 (25)	20.8 (27)
6A	85.7 (96)	85.1 (143)	79.2 (103)
carriage rate			
5A+	25.0 (14)	28.6 (24)	38.5 (25)
5A−	75.0 (42)	71.4 (60)	61.5 (40)

AgP (A), aggressive periodontitis patients; ChP (C), chronic periodontitis patients; H, healthy subjects; MMP, matrix metalloproteinase; SNP, single nucleotide polymorphism.
No statistically significant differences were observed among all groups.

be quite different from those previously reported in European Caucasians (Zhu et al. 2001, Ghilardi et al. 2002, Lamblin et al. 2002). That is, MMP-3 5A/5A genotype distribution was ca. 4–10 times higher in the Caucasians than in the Japanese. Likewise, MMP-1 2G/2G genotype distribution was approximately two-fold higher in the Japanese than in the Caucasians.

Additionally, MMP-1 or MMP-3 SNPs have been studied in various diseases in several populations. Although some papers found significant differences between diseased and healthy individuals (Kanamori et al. 1999, Terashima et al. 1999, Nishioka et al. 2000, Zhu et al. 2001, Ghilardi et al. 2002, Hinoda et al. 2002), the others showed no differences (Johnson et al. 2001, Ye et al. 2001, Zhang et al. 2001, Humphries et al. 2002, Lei et al. 2002). Therefore, the results are contradictory as to the influence on the susceptibility to diseases in some different races.

Very recently, De Souza et al. (2003) reported that MMP-1 2G allele was associated with severe ChP patients in Brazilian populations. Their result seems not in agreement with our report. The discrepancy may be explained by the fact that we analysed the genotype distribution and the allele frequency in Japanese individuals ($n = 384$) while the latter used Brazilian population ($n = 87$). As mentioned above, distribution of genotypes are often different in different races; for example, interleukin (IL)-1α (+4845) and IL-1β (−511, +3954) allele 2 were believed to be risk genotypes in adult periodontitis in a Caucasian population (Kornman et al. 1997). However, our previous report could not detect a significant difference in Japanese patients with AgP (Tai et al. 2002). Hodge et al. (2001) also failed to detect an association between IL-1 genotypes and generalised early onset periodontitis in another European Caucasian population. These findings imply that we should consider more carefully the genetic variation in different races and different disease types (e.g. generalised ChP, generalised or localised AgP).

Some of the possible reasons why MMP-1 and MMP-3 SNPs did not show a significant influence to the susceptibility to periodontitis in Japanese patients are discussed: the frequency of the assumed risk allele, MMP-1 2G was basically high even in Japanese healthy control group. It may be related that MMP-1 (fibroblast-type collagenase), not MMP-8 (neutrophil collagenase) or MMP-13 (collagenase-3) has important functions not only in the destruction of connective tissues, but also in the homeostatic tissue remodelling or wound healing of the periodontal tissues (Birkedal-Hansen 1993, Kiili et al. 2002). If all of the resident or migrated

Table 4. Haplotype distribution of MMP-1 and MMP-3 SNPs

Subjects	Frequency (%)		
	AgP	ChP	H
1G-5A	23.0	14.9	14.4
1G-6A	18.9	17.8	22.2
2G-5A	0	1.7	1.8
2G-6A	58.1	65.6	61.6

AgP, aggressive periodontitis patients; ChP, chronic periodontitis patients; H, healthy subjects.

For statistical analysis, a 2×2 contingency table containing the estimated numbers of the 1G-5A and those with the others was made, and the data were analysed by the χ^2 test.

No statistically significant differences were observed ($p > 0.05$).

host cells carrying MMP-1 2G allele systemically accelerate MMP-1 gene transcription and protein over-expression, not only local periodontal destruction but severe systemic collagenolytic diseases such as rheumatoid arthritis, cancer metastases, ulcers and vascular diseases might as well be combined. Indeed, most of the typical periodontitis patients do not often suffer systemic collagenolysis, indicating that some local environmental factors are important for fully transcriptional activation of MMP genes, though some types of diseases such as common diabetes mellitus, cardiovascular diseases or inflammatory diseases are occasionally seen with periodontitis.

Thus, we may also need to consider that MMP gene transcription could be regulated by other many local factors such as cytokines, hormones, growth factors, bacterial metabolites, adhesion molecules and so on. There are also various regulatory steps involved in periodontal connective/bone tissue destructions; from bacterial infection into host cells, host immune responses including the cytokine networks, MMPs and tissue inhibitors of MMPs (TIMPs) regulations.

Furthermore, recent studies proved that several MMP members: MMP-2, -3, -7, -8, -11 or MMP-12 single gene knockout mice did not show any apparent disorders (Zhou et al. 2000). They explained that some MMP members share common extracellular matrix substrates and compensate these functions for each other. These shared functions indicate that a single gene polymorphism of an MMP may not have enough effect on disease susceptibility or progression.

Interestingly, the haplotype frequencies and the linkage disequilibrium results of MMP-1 and MMP-3 polymorphisms, which are both localised in 11q22.3 chromosome adjacent to each other, revealed that non-risk 1G and risk 2G alleles in MMP-1 were more frequently linked to the risk 5A and non-risk 6A alleles in MMP-3, respectively. These risk and non-risk linkage combinations could contribute to the functional compensation of MMP function, in other words, protective function of host homeostasis.

Nevertheless, most of the multifactoral diseases including AgP and ChP are influenced, at least in part, by genetic factors and other environmental factors. It is believed that the combination of several significant risk gene polymorphisms in certain individuals synergistically elevates a susceptibility to the disease (Michalowicz et al. 2000). Further studies are needed to search for other risk gene polymorphisms that confer susceptibility to periodontitis and may contribute to diagnosis of the disease. In the future, diagnostic periodontal risk gene assessments may be of assurance in the detection of periodontitis-susceptible patients.

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