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The prevalence and pathogenic differences of *Porphyromonas gingivalis fimA* genotypes in patients with aggressive periodontitis

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Background: The *fimA* gene, which encodes fimbrillin (FimA), is found in *Porphyromonas gingivalis* and has been classified into six genotypes based on nucleotide sequence. *P. gingivalis* that possesses the type II *fimA* gene is prevalent in adult periodontitis.

Objectives: The aim of this study was to investigate the prevalence of *P. gingivalis fimA* genotypes in Japanese aggressive periodontitis patients, and to examine their virulence.

Methods: Subgingival plaque samples were obtained from 223 sites in 18 aggressive periodontitis patients and 95 sites in 22 periodontally healthy young adults. *Actinobacillus actinomycetemcomitans*, *P. gingivalis* and *Tannerella forsythensis* detection, determination of the *fimA* genotype in *P. gingivalis*, and the quantification of *P. gingivalis* were analyzed by polymerase chain reaction (PCR) methods. The proteolytic activities of the *P. gingivalis fimA* type I and *fimA* type II were also examined.

Results: In aggressive periodontitis patients, the most prevalent *fimA* genotype was the type II (46.7%), followed by the type Ib and type I, whereas in healthy subjects, the type I *fimA* was the only genotype detected. The number of *P. gingivalis* pathogens was the greatest in the type I *fimA* positive sites, and the frequency of coexisting *A. actinomycetemcomitans and T. forsythensis* was highest in the type II *fimA* positive sites in the aggressive periodontitis patients. Both the arginine-specific cysteine proteinase (Arg-gingipain) and lysine-specific cysteine proteinase (Lys-gingipain) activity of the *P. gingivalis fimA* type I strain were significantly higher than those of the *fimA* type II strains.

Conclusions: These results suggest that differences in virulence exist among different *fimA* genotypes. Coadherence with other pathogens in *P. gingivalis fimA* type II-associated aggressive periodontitis and quantitative increases in *P. gingivalis* in *fimA* type I-associated aggressive periodontitis are related to this virulence.

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Early onset periodontitis has been described as a group of diseases characterized by rapid periodontal destruction and alveolar bone loss in teenage, adolescent and young adults (1, 2). Recently, early onset periodontitis was newly classified as aggressive periodontitis (3). The relationship between various putative periodontal pathogens and aggressive periodontitis has been extensively studied (1, 4, 5). Many studies have shown that a highly virulent microbiota is associated with aggressive periodontitis (5, 6). *Actinobacillus actinomycetemcomitans* is frequently detected in patients with localized aggressive periodontitis, whereas in generalized forms of aggressive periodontitis, which occur in slightly older subjects and affect most of the dentition, a different microbiota including *Porphyromonas gingivalis* and *Tannerella forsythensis* (formerly *Bacteroides forsythus*) has been suggested (5, 7–9).

P. gingivalis is considered one of the most important pathogens causing periodontal disease (10), and several studies have demonstrated a clonal heterogeneity in virulence among various *P. gingivalis* strains (11). Amano *et al.* reported that *P. gingivalis* can be classified into five genotypes based on the genomic diversity of the *fimA* gene that encodes fimbrillin, a structural subunit protein of fimbriae and an important virulence factor of *P. gingivalis* (12–14). Recently, Nakagawa *et al.* identified a new *fimA* gene variant, named type Ib (15). *P. gingivalis* strains possessing type II *fimA* were detected in a majority of *P. gingivalis*-positive adult periodontitis patients (13). These results suggest that *P. gingivalis* with type II *fimA* might be a disease-associated strain.

Quantitative increases in specific pathogens seem to be important for the initiation and progression of periodontal diseases. There are many studies that have detected subgingival periodontal pathogens and quantified the level of them in periodontally healthy or diseased sites using the polymerase chain reaction (PCR) method (16, 17). However, there is no research that has investigated the relationship of subgingival microbial flora and the *P. gingivalis fimA* genotype.

The present study investigated the prevalence of the *P. gingivalis fimA* gene in aggressive periodontitis patients compared with periodontally healthy young adults. The detection frequency of *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis*, and the level of *P. gingivalis* in aggressive periodontitis patients was also examined to elucidate the difference in pathogenicity of the *fimA* genotype. Moreover, the different proteolytic activities of *P. gingivalis* with type I *fimA* and type II *fimA* genotypes were determined.

Materials and methods

Subjects

A total of 18 aggressive periodontitis patients and 22 periodontally healthy individuals referred to the Department of Periodontology at the Dental Hospital, Kyushu University, were selected for this study. All aggressive periodontitis patients (seven males and 11 females, aged 19–34 years, mean 27.6) exhibited severe periodontal destruction; probing pocket depth was greater than 5 mm in multiple sites. The periodontally healthy controls (six males and 16 females, aged 19–31 years, mean 23.4) had healthy gingiva, no radiographic evidence of bone loss, and probing pocket depth less than 3 mm. No subjects had any significant medical history, systemic diseases, or disorders that might affect the outcome of the periodontal therapy, and those that had received either periodontal therapy for at least 1 year prior to the examination or antimicrobial medication during the last 3 months were excluded.

Clinical examinations and microbiological sampling

Probing pocket depth and bleeding on probing were recorded in all subjects at baseline. Probing pocket depth was measured to the nearest mm at six sites in each tooth using a conventional periodontal probe. The presence or absence of bleeding on probing was registered after probing.

Subgingival plaque samples were collected 1 week after the clinical examinations. Using sterile paper points, samples were randomly collected from sites with ≥4 mm of probing pocket depth in each aggressive periodontitis patient, and from sites with ≤ 3 mm of probing pocket depth in each control subject. Bacterial DNA in the subgingival plaque samples was purified by Insta Gene™ Matrix solution (Bio-Rad Laboratories, Hercules, CA, USA) in accordance with the manufacturer's protocol and stored at −80°C until use.

Polymerase chain reaction

A. actinomycetemcomitans, *P. gingivalis* and *T. forsythensis* detection and *P. gingivalis fimA* genotype determination were performed using PCR methods. PCR amplification was performed with a total volume of 50 μl consisting of 20 μl DNA mixture and 30 μl of reaction mixture containing 1 × PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM NaCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs (dATP, dCTP, dGTP, dTTP), 0.2 μM of each primer, and 2.5 U of Taq DNA polymerase. The PCR amplification reagents were purchased from Takara Shuzo Co. (Shiga, Japan). To determine the presence of *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis*, three sets of oligonucleotide primers were selected according to the nucleotide sequences of the *A. actinomycetemcomitans* JP2 *lktA* gene, *P. gingivalis* ATCC 53977 *prtC* gene and *T. forsythensis* 338 16S ribosomal RNA gene, respectively (18, 19). To determine the *P. gingivalis* with the type I through type V and type Ib *fimA* gene, type specific primers were constructed according to Amano *et al.* (13) and Nakagawa *et al.* (15). All primers were synthesized and labeled commercially by Takara Shuzo Co. PCR amplification was carried out in a thermal cycler with previously described cycle conditions (13, 15, 18).

Analysis of polymerase chain reaction products

The PCR products were analyzed by 1.2% agarose gel electrophoresis. Gels

were stained with ethidium bromide, and visualized and photographed using an Image Master video documentation system (UV Transilluminator, Toyobo, Osaka, Japan). The number of *P. gingivalis* was determined by colorimetric PCR assay as described previously (18).

Bacterial strains and growth conditions

P. gingivalis ATCC 33277 (*fimA* type I) and ATCC 53977 (*fimA* type II) were used and maintained on CDC anaerobic blood agar (Becton Dickinson, Cockeysville, MD, USA) in an anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2). To isolate the clinical strains of *P. gingivalis* possessing *fimA* type I and type II, subgingival plaque was collected, using sterile paper points, from two patients with progressive periodontal disease and cultured on CDC anaerobic agar in an anaerobic atmosphere. Single black-pigmented colonies were examined with the described procedure for the isolation of *P. gingivalis* and its *fimA* genotype. Finally we obtained two *P. gingivalis* strains with *fimA* type I (MPW 1-01) and *fimA* type II (MPW 2-01).

Enzymatic assays

The microorganisms were grown in brain heart infusion broth supplemented with hemin (5 μg/ml) and menadione (1 μg/ml) to the mid-logarithmic phase, then harvested by centrifugation. The supernatant was used for the enzymatic assay. Lysine-specific cysteine proteinase (Lys-gingipain) and arginine-specific cysteine proteinase (Arg-gingipain) activities were determined using synthetic substrates, *N*-*p*-Tosyl-Gly-Pro-Lys-*p*-nitroanilide (TGPLPNA: Sigma, St Louis, MO, USA) and *N*-α-benzoyl-DL-Arg-*p*-nitroanilide (BAPNA: Sigma), respectively (20). In brief, bacterial supernatants were added to reaction mixtures containing 0.25 mM BAPNA and 0.25 mM TGPLPNA for Arg-gingipain and Lys-gingipain, respectively. The reaction mixtures were incubated at 37°C for 2 h. After adding the samples, absorbance was measured at 405 nm on a spectrophotometer.

Table 1. Detection frequency of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* in aggressive periodontitis patients and healthy control subjects

Pathogen	No. of samples (%)	
	Aggressive periodontitis	Healthy
A. actinomycetemcomitans	76* (34.1)	6 (6.3)
P. gingivalis	178* (79.8)	16 (16.8)
T. forsythensis	205* (91.9)	16 (16.8)

*Significant difference between the aggressive periodontitis patients and healthy controls using chi-squared statistics ($p < 0.01$).

Statistical analysis

The comparative frequency of each *fimA* genotype in the aggressive periodontitis patients, the detection frequency of each of the three organisms (*A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis*) in the patients and controls, and the effect of coexistence of *A. actinomycetemcomitans* and *T. forsythensis* on each *fimA* genotype were analyzed by the chi-squared exact test or Fisher's exact test. The level of *P. gingivalis*, indicated as the site frequencies of quantitative ranges of each *fimA* type, were examined using a nonparametric Mann–Whitney's *U*-test. Proteolytic activity data were statistically analyzed using a *t*-test. In all statistical tests, a *p*-value of < 0.05 was considered statistically significant.

Results

A total of 223 sites in 18 aggressive periodontitis patients and 95 sites in 22 control subjects were examined in this study. First, the detection frequency of *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* in the aggressive periodontitis patients and control subjects was examined. The percentage of positive sites for each pathogen is shown in Table 1. The prevalences of *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* in the aggressive periodontitis patients were significantly higher than in the control subjects, respectively ($p < 0.01$).

Furthermore, 126 *P. gingivalis*-positive sites with probing pocket depth ≥4 mm from the aggressive periodontitis patients (randomly selected around eight sites from each patients) and 16 *P. gingivalis*-positive sites from the control group were used to investigate the detection frequency of the six *fimA* genotypes. As shown in Table 2, in the aggressive periodontitis group, type II *fimA* was found in 51 of the 126 sites (40.5%), followed by type Ib *fimA* in 30 sites (23.8%), and type I *fimA* in 29 sites (23.0%). On the other hand, type I *fimA* was the only genotype detected in the healthy subjects. Next, the number of *P. gingivalis* found was investigated to determine the relationship between the level of *P. gingivalis* and *fimA* genotypes. As shown in Fig. 1, in the sites with the type I *fimA* genotype, the level of *P. gingivalis* was significantly higher than in the sites harboring type II or

Table 2. Detection frequency of the *Porphyromonas gingivalis fimA* genotypes in the aggressive periodontitis and healthy subjects

fimA genotype	No. of samples (%)	
	Aggressive periodontitis	Healthy
fimA I	29 (23.0)	16 (100)†
fimA Ib	30 (23.8)	0 (0)
fimA II	51 (40.5)*	0 (0)
fimA III	10 (7.9)	0 (0)
fimA V	3 (2.4)	0 (0)
UN	3 (2.4)	0 (0)

*Significant difference among the *fimA* genotypes in the aggressive periodontitis patients using chi-squared statistics ($p < 0.05$).
†Significant difference among the *fimA* genotypes in the healthy subjects using Fisher's exact test ($p < 0.01$).
UN, untypeable samples.

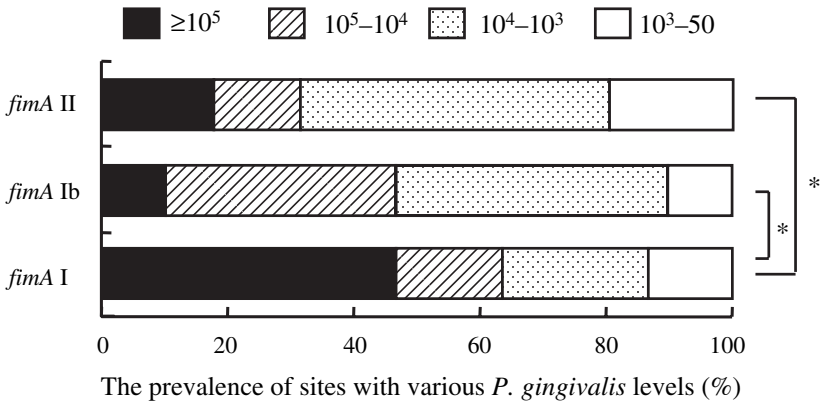

Fig. 1. Relationship between the level of *Porphyromonas gingivalis* and *fimA* genotypes found in the sites harboring *P. gingivalis* in aggressive periodontitis group. Stacked bar charts of the percentage of sites with different levels of *P. gingivalis* in each site with *fimA* type I, type Ib and type II, respectively. *P. gingivalis* level is indicated as the site frequencies of quantitative ranges. *Significant differences detected by Mann–Whitney's *U*-test ($p < 0.05$).

type Ib *fimA* genotypes in aggressive periodontitis patients ($p < 0.05$). The level of *P. gingivalis* in the type I *fimA* detected sites of the healthy subjects was mostly below 10^3 and significantly lower in any of the *fimA* genotype sites detected in the aggressive periodontitis patients ($p < 0.01$) (data not shown).

The detection frequency of *A. actinomycetemcomitans* and *T. forsythensis* in the sites harboring each *P. gingivalis fimA* genotype in aggressive periodontitis group was examined to investigate the relationship between *fimA* genotype and the coexistence of these pathogens with *P. gingivalis*. The coexistence of these pathogens was observed in 34 of the 51 sites with the type II *fimA* genotype (66.7%) and in 37.9% of sites with the type I *fimA* genotype, but coexistence was not detected at all in type Ib *fimA*-positive sites (results not shown). The detection frequency of this combination in type II *fimA* positive sites differed significantly from the sites with other *fimA* genotypes ($p < 0.05$).

Figure 2 shows the proteolytic activity in culture supernatants of *P. gingivalis* reference strains and clinical isolates possessing type I and type II *fimA* genotypes. Both the Arg-gingipain and Lys-gingipain activity of the *P. gingivalis* ATCC 33277 strain were significantly higher than that of the *P. gingivalis* ATCC 53977 strain ($p < 0.01$). Similar results were found between *fimA* type I (MPW 1-01) and type II (MPW 2-01) strains of *P. gingivalis* clinical isolates.

Discussion

This study examined the prevalence of *P. gingivalis fimA* genotypes and the pathogenic differences in *P. gingivalis* among the *fimA* genotypes in aggressive periodontitis patients. The results show that in the aggressive periodontitis patients, the most prevalent *fimA* genotype of *P. gingivalis* is type II, followed by type Ib and type I, respectively. Almost half the aggressive periodontitis patients possessed type II *fimA*. On the other hand, in the healthy young adults the prevalence of *P. gingivalis* with type I *fimA* was significantly higher. These results suggest a strong association between *P. gingivalis* possessing type II *fimA* and aggressive periodontitis. Several studies have shown that the type II *fimA* genotype of *P. gingivalis* is associated with periodontal disease (12–14). Amano *et al.* reported that the majority of adult periodontitis patients harbor type II *fimA* and that the next most prevalent

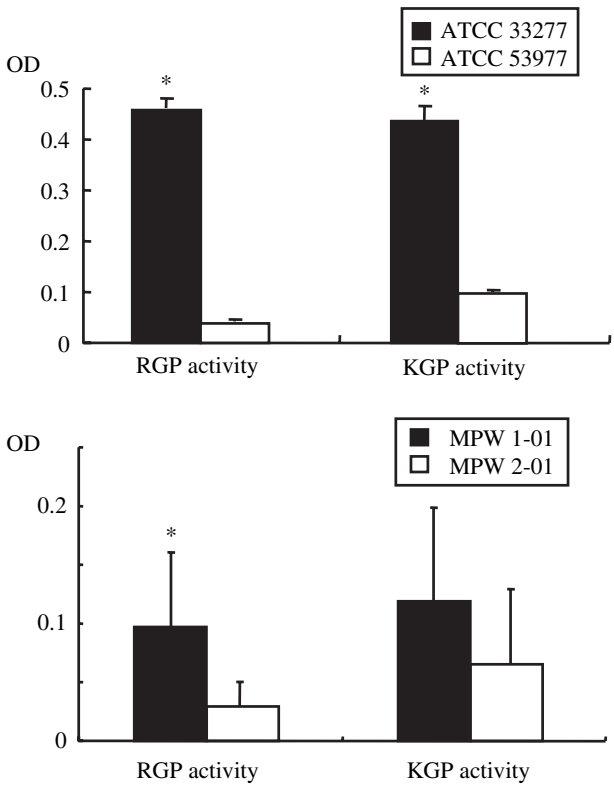

Fig. 2. Differences in the Arg-gingipain (RGP) and Lys-gingipain (KGP) activity of *Porphyromonas gingivalis* with the type I *fimA* and the type II *fimA*. ATCC 33277, *fimA* type I; ATCC 53977, *fimA* type II; MPW 1-01, *fimA* type I; MPW 2-01, *fimA* type II. *$p < 0.05$ according to the *t*-test.

type is type IV (12, 13). They also showed that the most prevalent *fimA* genotype in healthy subjects is type I. The prevalence of type II *fimA* was lower and that of type I was higher, respectively, in the aggressive periodontitis patients observed in the present study compared with the results of previous reports.

Fimbriae are thought to play an important role in the colonization and bacterial invasion of periodontal tissues (10). Nakagawa *et al*. reported that *P. gingivalis* with type II *fimA* adhered to and invaded epithelial HEp-2 cells to a greater extent than the type I or type IV *fimA* (21). However, *P. gingivalis* with type I *fimA* exhibited a marked ability to adhere to and invade these cells in their study, although the ability of *P. gingivalis* possessing type I *fimA* was less than that of those possessing type II. On the other hand, *P. gingivalis* expresses a number of potential virulent factors other than fimbriae.

Proteolytic enzymes are known to degrade a number of physiologically significant proteins and then destruct the host tissues. They are also related to other important biological activities, such as escaping a host's defense mechanisms, affecting a host's vascular systems, processing bacterial host proteins, and hemagglutinin activities (10, 22). *P. gingivalis* strains possessing type I *fimA* (ATCC3327, 381) or type IV *fimA* (W50, W83) have mostly been used in research concerning *P. gingivalis* proteinase (11). Nasser *et al*. reported that the interaction of *P. gingivalis* possessing type I *fimA* with endothelial cells and the subsequent activation of a proinflammatory response are mediated both by fimbriae and proteinase mechanism (23). Recently, it has been suggested that *P. gingivalis* is a predominant periodontopathic bacterium and that it is strongly associated with aggressive periodontitis patients in Japanese populations (24). The present study revealed that *P. gingivalis* with type I *fimA* (ATCC 33277, MPW 1-01) has higher Arg-gingipain and Lys-gingipain activities in culture supernatants than *P. gingivalis* with type II *fimA* (ATCC 53977, MPW 2-01). Recently, we also examined both proteinase activities in cell extracts of *P. gingivalis* clinical isolates and found that Arg-gingipain and Lys-gingipain activities of type I strains were significantly higher than type II strains (data not shown). The level of *P. gingivalis* in the sites with type I *fimA* was significantly higher than in those harboring other *fimA* genotypes in this study. In addition, the level of *P. gingivalis* with type I *fimA* in the healthy subjects was significantly lower than in the sites with type I *fimA* in the aggressive periodontitis patients ($p = 0.00027$). The increased number of periodontal pathogens is also thought to be important for the initiation and progression of periodontal disease (25). Even though *P. gingivalis* with type I *fimA* exhibited a weaker ability to adhere to and invade host tissues compared to those with the type II *fimA* strain, the increased number of *P. gingivalis* possessing type I *fimA* might facilitate the action of both fimbriae and proteinase. Therefore, we suggest that the high levels of *P. gingivalis* possessing type I *fimA* might be associated with aggressive periodontitis.

A. actinomycetemcomitans, *P. gingivalis* and *T. forsythensis* are thought to have a particular significance in various forms of severe periodontal disease (8, 9). Each of these three pathogens was frequently detected in the diseased sites of the aggressive periodontitis patients in the present study. Gmür *et al*. showed that the combination of *P. gingivalis* and *T. forsythensis* was strongly related to periodontal disease initiation and progression (26). They also described a strong relationship between *P. gingivalis* and *T. forsythensis* in subgingival plaque samples. In the present study, the coexistence of *A. actinomycetemcomitans* and *T. forsythensis* in the sites harboring *P. gingivalis* with type II *fimA* was significantly high. We investigated the coaggregation of *P. gingivalis* with *T. forsythensis* and found that the coaggregation potential of type II *fimA* strains was significantly higher than that of type I *fimA* strains (data not shown). These suggest that *P. gingivalis* possessing type II *fimA* might facilitate the coadherence of other bacteria and contribute to the formation of a biofilm. On the other hand, the new type Ib *fimA* identified by Nakagawa *et al*. reportedly shows a 97.1% homology with type I *fimA* (15). Since the type Ib *fimA* is the most newly detected genotype, there are no studies about the *fimA* type Ib strains. Now we are trying to isolate the type Ib *fimA* clinical strain to investigate proteinase activity and coaggregation with *T. forsythensis*. In the present study, in the sites harboring *P. gingivalis* with type Ib *fimA*, *A. actinomycetemcomitans* was not detected. However, the number of patients in this study was too small to indicate the correlation between *P. gingivalis* with type Ib *fimA* and *A. actinomycetemcomitans*. Further research is therefore required to elucidate this discrepancy.

In conclusion, *P. gingivalis* possessing type II *fimA*, type I *fimA* or type Ib *fimA* is strongly associated with aggressive periodontitis. *P. gingivalis* with type II *fimA* might facilitate the coadherence of other pathogens, and the quantitative increase of *P. gingivalis* with type I *fimA* might enhance its pathogenicity. Further research is necessary to clarify the pathogenicity of *P. gingivalis* with type Ib *fimA*.

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