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Association of the invasion ability of *Porphyromonas gingivalis* with the severity of periodontitis

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Porphyromonas gingivalis is one of the well-characterized periodontal pathogens involved in periodontitis. The invasive and proteolytic activities of *P. gingivalis* clinical isolates have been shown to be associated with heterogenic virulence, as determined in a mouse abscess model. The aims of the present study were to identify a *P. gingivalis* strain with a low virulence among clinical isolates, based on its invasive ability and cytokine proteolytic activities, and to explore the preferential degradation of a certain cytokine by *P. gingivalis*. *P. gingivalis* ATCC 33277, W50, and 10 clinical isolates were used. After incubating bacteria with IL-4, IL-6, IL-10, IL-17A, TNFα, IFNγ, and IL-1α, the amounts of remaining cytokines were determined by ELISA. Invasion ability was measured by a flow cytometric invasion assay. There was inter-strain variability both in the cytokine proteolytic activities and invasion ability. In addition, differential degradation of cytokines by *P. gingivalis* was observed: while IFNγ and IL-17A were almost completely degraded, inflammatory cytokines TNFα and IL-1α were less susceptible to degradation. Interestingly, the invasion index, but not cytokine proteolytic activities, of *P. gingivalis* had strong positive correlations with clinical parameters of subjects who harbored the isolates. Therefore, the invasive ability of *P. gingivalis* is an important virulence factor, and the bacterial invasion step may be a good target for new therapeutics of periodontitis.

Introduction

Periodontitis is a chronic inflammatory disease caused by the interactions between the subgingival biofilm and host immune responses, which lead to the destruction of tooth-supporting tissues. Periodontitis is characterized as a polymicrobial disease that involves dysbiosis of the indigenous flora in the subgingival biofilm.[1] Among the more than 500 bacterial species harbored in the subgingival biofilm, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* are well-characterized periodontal pathogens.[2]

P. gingivalis, a gram-negative obligate anaerobe, has a number of virulence factors such as cysteine proteases (gingipains), major fimbriae, lipopolysaccharide, and capsule.[3] Among the virulence factors of *P. gingivalis*, gingipains, which consist of Arg-gingipain (Rgp) and Lys-gingipain (Kgp), can degrade a number of host proteins, including cytokines/chemokines, immunoglobulins, complement proteins, and host cell receptors.[4,5] The degradation of various cytokines, including IL-1β, IL-4, IL-5, IL-6, IFNγ, and TNFα, by purified gingipains or live *P. gingivalis*[6-10] suggests a possibility that *P. gingivalis* may be able to modulate the immune response through preferential inactivation of a certain cytokine. However, the degradation of these cytokines has not been simultaneously studied.

P. gingivalis fimbriae are filamentous appendages that mediate the bacterial adherence to various host cells, other bacteria, and host macromolecules.[11] The *fimA* gene, which encodes FimA (a subunit of major fimbriae), has been classified into 6 types (I-V and Ib) based on the nucleotide sequences.[11] While type II, IV, and Ib genotypes are associated with periodontitis, the type I genotype is associated with periodontal health.[12,13] In particular, *P. gingivalis* isolated from patients mainly contain the *fimA* genotype II.[12,14]

P. gingivalis has been shown to invade various host cells *in vitro*, including gingival epithelial cells, endothelial cells, and gingival fibroblasts.[15-17] In addition, *P. gingivalis* has been detected within gingival tissues obtained from patients with periodontitis, suggesting an important role of tissue invasion in the pathogenesis of periodontitis.[18,19]

Inaba et al.[20] reported that the invasive and proteolytic activities of *P. gingivalis* clinical isolates are associated with

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heterogenic virulence, which has been studied in the mouse abscess model. Although probiotics have only included commensal bacteria and shown their usefulness as an adjunct therapy in chronic periodontitis,[21,22] *P. gingivalis* strains with a low virulence may be also used for the prevention of colonization or replacement of highly virulent *P. gingivalis*. The aims of the present study were to identify a *P. gingivalis* strain with a low virulence among clinical isolates based on its invasive ability and cytokine proteolytic activities, and to explore the preferential degradation of a certain cytokine by *P. gingivalis*.

Results

Ten *P. gingivalis* clinical isolates were isolated: one (KUMC-H1) from a periodontally healthy subject and 9 (KUMC-P1∼KUMC-P8 and KUMC-P10) from periodontitis patients. The demographic and clinical characteristics of the study subjects are summarized in **Table 1**. Patient P6 had a past history of tooth extraction (#16, #17, and #27) due to root caries below the crowns in 2008. Although the cause for the loss of the other teeth was not known, the levels of remaining alveolar bones were not substantially reduced compared to those of the adjacent tooth, suggesting a cause other than periodontitis. The causes for the tooth loss in patients P1, P7, and P8 were not available.

$CD4^+$ T-helper (Th) cell subsets, such as Th1, Th2, Th17, and Tregs, play a critical role in modulating the immune responses.[23,24] To investigate the possibility that *P. gingivalis* clinical isolates differentially modulate the immune response through the preferential degradation of a certain cytokine, a mixture of IFNγ, IL-4, IL-17A, TNFα, IL-6, and IL-10 (Th1, Th2, Th17, two inflammatory, and an anti-inflammatory cytokine, respectively) was incubated with viable *P. gingivalis*. The amounts of residual recombinant cytokines determined by ELISA revealed a substantial inter-strain difference in the degradation of IFNγ ($p = 0.007$), IL-17A ($p = 0.007$), TNFα ($p = 0.000$), IL-6 ($p = 0.004$), and IL-10 ($p = 0.018$). In the case of the laboratory strains, W50 tended to have a higher degradation ability than ATCC 33277 for 4 cytokines. In general, KUMC-P1 and KUMC-P8 strains showed reduced degradation of all tested cytokines compared with other strains (**Fig. 1A**). Interestingly, TNFα was less susceptible to degradation by *P. gingivalis.* In particular, W50, KUMC-P2, KUMC-P5, and KUMC-P6 strains showed significant differences in the degradation rate between TNFα and all other cytokines, suggesting the preferential protection of an inflammatory cytokine. Therefore, the ability of *P. gingivalis* to degrade IL-1α, another inflammatory cytokine, was examined. Interestingly, unlike other cytokines, IL-1α was resistant to degradation by *P. gingivalis*, which showed only 3 to 13% degradation rate with no significant inter-strain difference (**Fig. 1B**). Taken together, there was inter-strain variability in the ability to degrade cytokines and the inflammatory cytokines TNFα and IL-1α were less susceptible to degradation by all strains of *P. gingivalis*.

The invasion of *P. gingivalis* clinical isolates into HOK-16B cells was examined by a flow cytometric invasion assay. *P. gingivalis* ATCC 33277 and W50 strains were used as controls. In the case of the laboratory strains, the invasive ability of *P. gingivalis* ATCC 33277 was greater than that of W50, as was reported by another group[25]; this difference, however, was not significant. All 10 clinical isolates were able to invade HOK-16B cells. Among them, the invasion indexes of KUMC-P7, KUMC-P8, and KUMC-P10 strains were substantially higher than those of other strains, which resulted in an approximately 20-fold difference between the lowest and the highest strains (**Fig. 2**).

We examined whether the invasive abilities or proteolytic activities of the *P. gingivalis* clinical isolates have a relationship to any clinical parameters. Unexpectedly, the invasion index of *P. gingivalis* had strong positive correlations with the mean probing depth (PD, $r = 0.745$, $p = 0.013$), mean marginal bone loss ($r = 0.794$, $p = 0.006$), and % of sites with PD greater than 5 mm ($r = 0.903$, $P < 0.0005$) (**Fig. 3**), but not with those of the site from which *P. gingivalis* was isolated. These clinical parameters were not associated with degradation of any cytokine.

Because *fimA*–disrupted mutants have been shown to lose almost all of their invasive ability,[26] we analyzed the entire coding sequence of the *fimA* gene to determine an underlying mechanism for the heterogenic invasive ability of *P. gingivalis*. A sequence similarity search revealed that KUMC-P10 has a type I genotype, while all the other strains have type II. The translated sequences of KUMC-P4 and KUMC-P7 *fimA* genes were identical to those of TDC222 and 7680 strains in the database, respectively. KUMC-P2 and KUMC-P3 had identical FimA sequences. Far from our expectations, the strains with high

Table 1. The demographic and clinical characteristics of study subjects.

	H1	P1	P2	P3	P4	P5	P6	P7	P8	P10
Age (years)	41	64	48	55	47	35	50	58	46	47
Sex	F	M	M	M	M	M	F	F	M	M
Total tooth count	28	27	28	28	28	28	20	24	27	28
Mean PD (mm)	2.46	2.95	3.77	3.39	3.27	3.38	2.79	3.97	4.38	4.58
Sites with PD > 5 mm (%)	0	12.3	20.8	5.4	9.5	14.3	2.5	18.8	40.1	32.7
Mean marginal bone loss (%)	6.84	33.13	38.24	24.11	25.55	27.21	18.48	34.79	33.42	49.49
PD of *Pg*-isolated site (mm)	2	6	10	10	10	5	5	12	10	10
Bone loss of *Pg* -isolated tooth (%)	5.4	46	69	58	67	27	20	67	72	38
Smoking	N	N	N	N	N	C	N	N	P	P

M: male, F: female, PD; probing depth, H: isolated from healthy subject, P: isolated from periodontitis patient, N: never, C: current, P: previous.

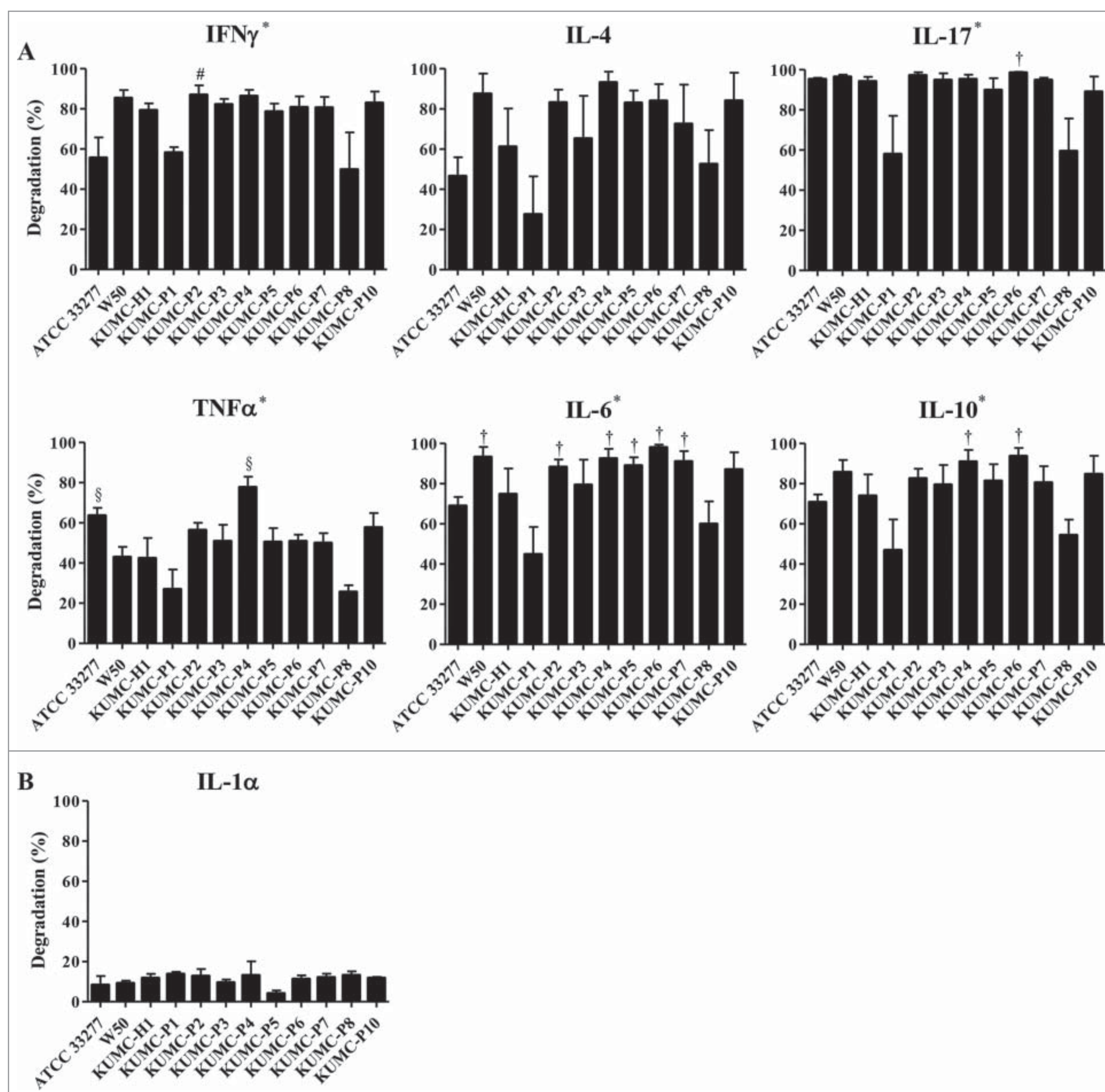

Figure 1. Various proteolytic activity of *P. gingivalis* strains. (**A**) A mixture of recombinant cytokines (IL-4, IL-6, IL-10, IL-17A, IFNγ, and TNFα) or (**B**) IL-1α were prepared in KGM medium, and incubated with viable *P. gingivalis* at 37°C for 1 h. After incubation, the supernatants were collected and the amount of remaining cytokines was determined by ELISA. The results indicate degradation percentage compared with control samples incubated without *P. gingivalis*. *$P < 0.05$ significant difference among strains, †$P < 0.05$ versus KUMC-P1, #$P < 0.05$ vs. KUMC-P8, §$P < 0.05$ versus both KUMC-P1 and KUMC-P8.

invasive ability (KUMC-P7, KUMC-P8, and KUMC-P10) were not clustered together in the phylogenetic tree (**Fig. 4A**). In the aligned FimA sequences, we could not identify any area where the amino acid sequences were shared by KUMC-P7, KUMC-P8, and KUMC-P10 but not by the others (**Fig. 4B**).

Discussion

In this study, we investigated the invasion ability and cytokine degradation by various *P. gingivalis* clinical isolates in order to identify a strain with a low virulence. Substantial inter-strain variability was observed both in the cytokine degradation and invasiveness, as reported by other groups.[20,25,27-29] Although a positive correlation of invasive efficiency with inflammatory parameters determined in the mouse abscess model has been reported,[20] a strong correlation between the invasion efficiency and the clinical indexes of patients who harbor the *P. gingivalis* strain is a novel and unexpected finding. Considering the ecological complexity of subgingival biofilm, the fact that a single virulence parameter of one species reflects disease severity is unexpected. It has been reported that *P. gingivalis* degrades

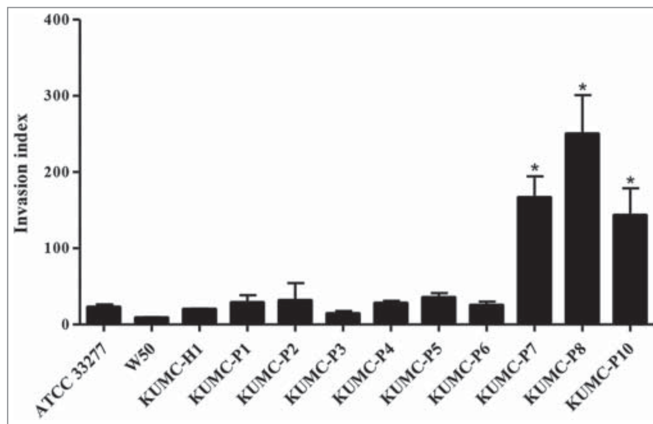

Figure 2. Various invasive ability of *P. gingivalis* strains. HOK-16B cells (6×10^4 cells/well) were seeded into 24-well plates and incubated with viable CFSE-labeled *P. gingivalis* at the MOI of 1000 for 4 h. After quenching the fluorescence of bacteria bound on the cell surface with trypan blue, the fluorescence of HOK-16B cells containing intracellular bacteria was analyzed by flow cytometry. $^*P < 0.05$ KUMC-P7, KUMC-P8, and KUMC-P10 have a significant difference compared with rest of strains.

paxillin and focal adhesion kinase after invasion into epithelial cells, which inhibits cellular migration and proliferation.[30] Therefore, one possible explanation is that microulceration and delayed wound healing caused by invasive *P. gingivalis* may contribute to the induction of changes in subgingival microbial composition, which in turn may contribute to the progression of periodontitis. This explanation is in line with the recent concept of the 'keystone pathogen' that low abundance pathogens such as *P. gingivalis* can induce dysbiosis of normally benign microbiota.[31]

Another controversial issue is in regards to the clonality of *P. gingivalis* within an individual. The detection of intra-individual clonal diversity by multilocus sequence typing changed the general concept that patients are colonized with a single strain of *P. gingivalis*.[32] A sensitive culture-independent method has detected *P. gingivalis* in the healthy sites of patients as well as in the diseased sites. It has been proposed that the virulence of *P. gingivalis* clones found in the healthy sites is different from those found in the diseased sites.[32] In our study, however, the invasive ability of *P. gingivalis* did not correlate with the clinical indexes of the sites from which *P. gingivalis* were isolated, but did correlate with the mean indexes. This contradicts the hypothesis that the disease development is determined by the heterogenic pathogenicity of *P. gingivalis* within a patient. There is a possibility that multiple clones detected within an individual are genetically related to each other in terms of pathogenicity. Otherwise, the multiple clones may be genetically diverse, but functionally similar; the Human Microbiome Project identified ubiquitous functional pathways among individuals despite the high degree of inter-individual variability in microbial taxa.[33] The variable amounts of subgingival biofilm and *P. gingivalis*, rather than heterogenic pathogenicity of *P. gingivalis*, may be an important determinant of periodontal status within a patient. The fact that *P. gingivalis* was sampled from a single site was a clear limitation of the study to extrapolate results on a patient level. Further studies using isolates from the multiple sites of each individual are warranted to clarify this issue.

Aside from the invasive ability, the ability of *P. gingivalis* to degrade cytokines did not show an association with any clinical parameters. However, the pathogenic heterogeneity of *P. gingivalis* determined in the mouse abscess model had a positive correlation with the activity of secreted Kgp gingipains.[20] This discrepancy may be attributed to the fact that the bacteria were subcutaneously injected in the mouse abscess model, bypassing the need of tissue invasion. In addition, proteases other than Kgp gingipains may be involved in cytokine degradation. The degradation of TNFα, IFNγ, and IL-4 by Rgp gingipain has been reported.[6,10,34] Another interesting finding was the nearly complete degradation of IFNγ and IL-17A along with the relative resistance of TNFα and IL-1α to degradation by *P. gingivalis*. The lower rate of IL-1β degradation compared to that of IL-6 has been also reported.[8] The degradation of Th1 and Th17 cytokines may contribute to the persistency of *P. gingivalis* infections, while the protection of inflammatory cytokines may contribute to the induction of chronic inflammation in gingival tissue. It is noteworthy similar resistance of IL-1α to degradation by *T. denticola*.[35]

The analysis of the *fimA* gene sequences revealed that the majority of *P. gingivalis* isolated from patients have type II *fimA* genotype, which coincides with a previous report.[12] However, the type II *fimA* genotype was not associated with a high invasive ability of *P. gingivalis*. This fact also coincides with a previous report that

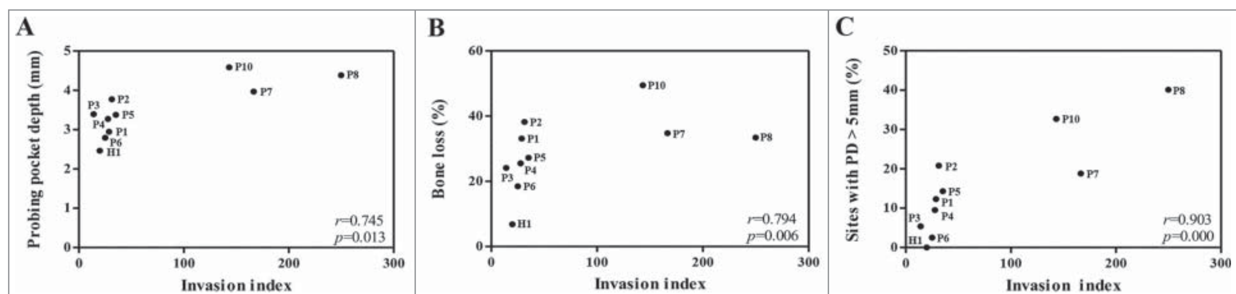

Figure 3. Strong positive correlations between clinical parameters of 10 subjects and the invasive ability of *P. gingivalis* strains. Two-tailed Spearman's rho correlations of the invasive ability of *P. gingivalis* with 3 clinical parameters (PD, marginal bone loss, and sites with PD > 5 mm) of subjects are shown.

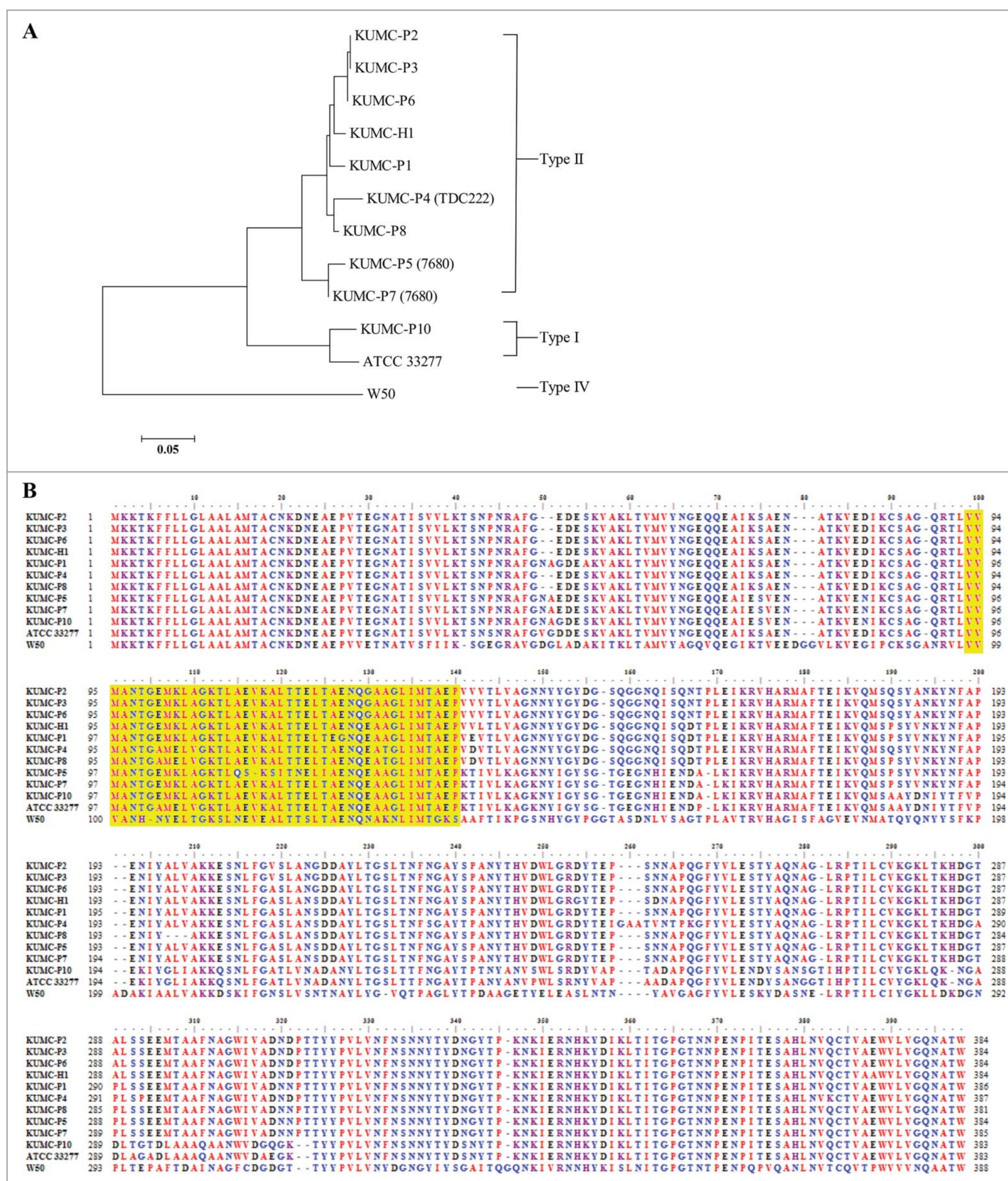

Figure 4. Analysis of *P. gingivalis fimA* sequences. Using the genomic DNA from 10 *P. gingivalis* clinical isolates, the entire coding area of *fimA* gene was amplified, sequenced, and translated into amino acid sequences. (**A**) A phylogenetic tree was constructed using the translated FimA sequences of 10 clinical isolates and 2 laboratory strains from the database by the neighbor-joining method. The scale bar represents genetic distance. (**B**) The translated amino acid sequences were aligned and yellow shaded area represents the epithelial binding domain of *P. gingivalis* fimbriae.[40]

the *fimA* genotypes of *P. gingivalis* are not related to the adhesion and invasion abilities.[29] We failed to identify a determinant of the high invasion ability within the primary sequence of FimA; this suggests that the invasion ability of *P. gingivalis* might be determined by the 3-D structure of FimA. Gingipains are also known to be involved in the adhesion/invasion of *P. gingivalis* through maturation of fimbriae and direct interaction with epithelial cells via the adhesin domain of gingipains.[36,37]

KUMC-H1, a clone that was isolated from the healthy subject, had the second lowest invasion ability. However, the relatively young age (41 year) of H1 among the subjects in the study may have contributed to the healthy status. KUMC-P1 is an interesting clone in terms of its both low invasion and low proteolytic abilities. The P1 patient, who was 64 years old, showed relatively shallow PD and low percentage of PD greater than 5 mm. A limitation of the current study is a small sample size to adjust for other risk factors, such as age, smoking, and oral hygiene. Compared to the traditional invasion assay (based on colony-forming unit counts), the flow cytometric invasion assay is easy and fast. Future studies involving larger sample sizes and the adjustment of other risk factors are required to assess the true risk of *P. gingivalis* invasive ability in periodontal health status.

In conclusion, the invasion of epithelial cells but not the degradation of cytokines by *P. gingivalis* was associated with clinical parameters of periodontitis, which indicates that the invasive ability of *P. gingivalis* may be an important virulence factor. In addition, the bacterial invasion step may provide a good target for new therapeutics of periodontitis.

Materials and Methods

Study subjects

A total of 10 adult subjects who sought dental treatment at the Anam Hospital of Korea University were recruited for the study. The study protocol was approved by the Institutional Review Board for Human Subjects of the Korea University Anam Hospital (IRB No. ED10053). Written informed consent was obtained from all individuals. Exclusion criteria were pregnancy, diabetes, and other systemic conditions that could affect the periodontal status. PD was measured at 6 sites per each tooth. The amount of marginal alveolar bone loss was estimated as the percentage of the length from the cement-enamel junction to the alveolar crest relative to the length of the root on panoramic view. One periodontally healthy subject exhibited no sites with PD > 4 mm and no marginal bone loss $> 20\%$. The nine subjects with periodontitis exhibited at least 2 or more sites with PD ≥ 5 mm and 2 or more teeth with marginal bone loss $> 20\%$ in one jaw.

Isolation and identification of *P. gingivalis* from clinical plaque samples

To isolate *P. gingivalis*, subgingival plaque samples were collected with a sterile paper point (Caulk-Dentsply) from the healthy site of a healthy subject and the diseased sites of periodontitis patients. The paper point was then placed in a sterilized dental transport medium. The plaque dispersed in the transport medium was streaked onto sheep blood agar with hemin and menadione (Sigma–Aldrich). The plates were incubated under an anaerobic atmosphere (10% CO_2, 5% H_2, and 85% N_2). After incubation, black-pigmented colonies were selected and anaerobically grown in a brain heart infusion broth (BD Diagnostic Systems) that was supplemented with 5 μg/ml hemin and 5 μg/ml menadione. The genomic DNA from cultured bacteria was used for PCR with 16S rRNA-based *P. gingivalis*-specific primers.[38] After confirming the *P. gingivalis* 16S rRNA gene sequence, bacterial stocks were stored in liquid nitrogen until further use. *P. gingivalis* ATCC 33277 (American Type Culture Collection), W50 (American Type Culture Collection), and 10 clinical strains of *P. gingivalis* were used and cultured under the same conditions.

Degradation of cytokines by *P. gingivalis* and ELISA

To examine the degradation of cytokines by *P. gingivalis,* a cytokine mixture of IL-4, IL-6, IL-10, IL-17A, TNFα, and IFNγ (Peprotech) were prepared in keratinocyte growth medium containing supplementary factors (KGM; Clonetics) without antibiotics at 33 pM (equivalent to 500 pg/ml of IL-4) and added to 24-well plates. Viable *P. gingivalis* (1×10^7cells/well) strains were added to a mixture of recombinant cytokines and incubated at 37°C for 1 h. After centrifugation, the residual cytokine concentration in the supernatants were determined using ELISA kits (R&D Systems) according to the manufacturer's instructions and were compared to that of the basal levels of controls, which were incubated with KGM alone. The degradation of IL-1α recombinant protein (500 pg/ml) (R&D Systems) was performed separately in a similar manner.

Cell culture and flow cytometric invasion assay

Immortalized human gingival keratinocyte HOK-16B cells were maintained in KGM. A flow cytometric invasion assay was performed as described previously.[39] HOK-16B cells were infected with 5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester (CFSE; Molecular Probe)-labeled bacteria at a multiplicity of infection (MOI) of 1000 in KGM without antibiotics for 4 h. Infected HOK-16B cells were washed with PBS and detached with trypsin-EDTA. After quenching the fluorescence of the bacteria bound on the surface with 0.4% trypan blue, the cells were analyzed using a FACSCalibur (BD Bioscience). For the negative controls, cells fixed with 3.7% formaldehyde were also exposed to the same amount of CFSE-labeled bacteria. In the flow cytometric invasion assay, the fluorescence intensity of host cells with invading bacteria is affected by the fluorescence intensity of labeled bacteria. The CFSE-labeling efficiency slightly varied from experiment to experiment, depending on the strains. To compare the invasive ability of different strains, the invasion index was calculated as follows: [mean fluorescence intensity (MFI) of infected cells-MFI of negative control cells]/ MFI of CFSE-labeled bacteria*100.

fimA sequence analysis

To determine the *fimA* gene sequences of *P. gingivalis* clinical isolates, the *fimA* genes were amplified by PCR using *fimA*-universal primers as previously described,[38] and the products were then sequenced (Cosmo Genetech). The obtained sequences were BLAST searched (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the database to find the closest *fimA* gene; and were translated using public software (http://www.fr33.net/). In addition, the translated amino acid sequences were aligned using the CLUSTAL W algorithm in the MEGA 6.0 (http://www.megasoftware.net/) and subjected to phylogenetic analysis by applying

neighbor-joining methods in the MEGA 6.0. The *fimA* gene sequences were registered in GenBank (GenBank accession numbers: KM987010~987019).

Statistical analysis

All data were expressed as the mean plus standard error of the mean (SEM) of the 3 experiments. Because the measured values were not normally distributed, nonparametric statistical methods were used. Inter-strain differences were analyzed by Kruskal-Wallis with the post-hoc tests. The correlations between the clinical parameters of 10 subjects and the invasive ability or proteolytic activity of *P. gingivalis* clinical isolates were determined by Spearman's rank correlation coefficient. All analyses were performed by 2-tailed tests using SPSS18.0 (SPSS Inc.). Data were considered statistically significant at P value of <0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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