The Effect of Porphyromonas Gingivalis FimA Genotypes in Subgingival Deposit on Clinical Parameters of Periodontitis

Sodsi Wirojchanasak[1*], Atittaya Chaowthawee[2], Sasikarn Krutkham[2], Teerawat Takamtiang[2], Watcharakorn Ngamsaad[2], Ichaya Yiemwattana[3], Jantipa Jobsri[4]

1. Section of Periodontics, Department of Preventive Dentistry, Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand.
2. Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand.
3. Section of Periodontics, Department of Preventive Dentistry, Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand.
4. Department of Oral Biology, Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand.

Abstract

Porphyromonas gingivalis (Pg) plays critical roles in the pathogenesis of periodontitis with varying virulence based on fimA gene variation. This study aimed to evaluate the correlation between the presence of Pg-specific fimA genotypes and clinical manifestations of periodontitis.

Clinical periodontal parameters were examined in 7 healthy periodontal controls and 21 periodontitis volunteers. Subgingival biofilms from volunteers were collected and randomly selected to isolate genomic DNA. Pg and Pg with specific fimA type2 genotype (PgfimAII) were detected utilizing a conventional polymerase chain reaction technique.

Periodontitis samples were categorized into 3 groups including PgfimAII positive (42.9%), other strains of Pg positive (38.1%) and Pg negative (19%) groups. No healthy controls contained Pg positive sites. Clinical attachment loss and pocket depth of PgfimAII group were different from Pg negative group (p<0.05). All diseased groups exhibited moderate to strong correlation with clinical attachment loss, pocket formation, and calculus amount at different levels.

In conclusion, clinical attachment loss, pocket formation, and calculus amount were linked to periodontitis with or without any Pg genotypes. The presence of studied bacteria did not correspond strongly to bleeding on probing or gingival index. PgfimAII contributed to severe attachment loss and deep pocket in different patterns from Pg negative group.

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Introduction

Periodontitis is a poly-microbial oral infectious disease at tooth supporting structures. Pathogenesis of periodontitis initiates from gingival texture change to pocket formation with advanced tissue destruction which can lead to tooth loss, impacting on function, esthetic and general health[1-2]. Severity of periodontitis was range from mild to the most severe conditions based on levels of clinical attachment loss (CAL), pocket depth (PD), and radiographic bone loss[3].

Predominant etiological factor of periodontitis is specific microflora in plaque biofilms that attach to tooth surface at subgingival area[4]. Porphyromonas gingivalis, a gram negative strictly anaerobe, is a key infectious pathogen contributing to development and progression of periodontitis[5]. One critical virulence factor of Pg is fimbriae, a cell-surface structure, that plays roles in attachment with other bacteria in biofilms and adherence to host cells[6]. Major subunit of fimbriae, fimbrillin, is encoded by fimA gene. The diversity of fimA genotypes can be used to classify Pg into 6 types (typeI-V and Ib) which are likely to contain various virulent capacities[7-8].

Functional studies and animal infection models showed varying adherence abilities and antigenic properties in Pg possessing different fimA genotypes[9-11]. Several clinical studies suggested that PgfimAII was a major strain contributing to periodontitis condition[8,12-14]. However, correlation with clinical periodontal status among different Pg genotype discrepancies were not conclusive. Our present

Corresponding author:
Sodsi Wirojchanasak,
Section of Periodontics, Department of Preventive Dentistry.
Faculty of Dentistry, Naresuan University
Phitsanulok 65000, Thailand.
E-mail: sodsiw@nu.ac.th

study aimed to investigate the association between the presence of different fimA-specific Pg genotypes and clinical manifestations of periodontitis.

Materials and methods

Human ethics approval was obtained from Naresuan University Ethical Committee, Phitsanulok, Thailand (IRB Number 0536/60). From 2017 to 2018, volunteers with $\geq$ 15 teeth who sought periodontal treatment at Periodontics Clinic, Dental Hospital, Faculty of Dentistry, Naresuan University were asked to sign an informed consent prior to the study participation.

All recruited periodontitis subjects were diagnosed as periodontitis with PD $\geq$ 4 mm and CAL $\geq$ 2 mm according to their clinical and radiographic characteristics. Control subjects were diagnosed as periodontal health without any pocket formation or CAL. Subjects with systemic conditions associated with periodontal responses including immunodeficiency, diabetes, pregnancy, other conditions that required antibiotic prophylaxis and intake of gingival enlargement inducing medicines were excluded from the study. Subjects did not smoke or receive periodontal treatment 6 months prior to the baseline examinations.

Clinical periodontal status was assessed by calibrated examiners at the baseline visit. The examinations included PD, CAL, Gingival index (according to Löe and Silness), bleeding upon probing (BOP), and calculus index (according to Greene and Vermillion Simplified oral hygiene index) with UNC-15 probe (Hu-Friedy, USA). The PD, CAL, and BOP was measured 6 sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual).

Specimens were taken at the following visit before scaling and root planing procedures. The collected areas were isolated with cotton pellets then supragingival deposits were removed with sterile gauzes. A sterile Mini-Five® Gracey curette (Hu-Friedy, USA) was inserted into the deepest part of pocket of all teeth with PD then removed once to collect subgingival biofilms. Subgingival biofilms from healthy controls were collected from all central incisor and molar teeth. The collected samples were transferred into a 1.5-ml tube containing 500-µl phosphate buffer saline solution and immediately transported on ice to the laboratory. The samples were stored at -20°C until they were used for molecular analysis.

The samples were randomized two sites from each periodontitis volunteer from both jaws and one site from each healthy periodontium volunteer. Total bacterial DNA from the selected samples were extracted using a NucleoSpin® Tissue XS genomic DNA extraction kit (Macherey Nagel, Germany) according to the manufacturer's instructions. Quality and concentration of all DNA samples were verified by NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, USA). Each obtained DNA sample was performed specific gene analysis. Polymerase chain amplification of 16S rRNA gene specific to Pg and fimA genotype 2 of Pg was used to analyze all strains of Pg and PgfimAII. Sterile deionized water without template DNA was used as a negative control. Plasmid pTZ57R/T vectors containing nucleotide sequences homologous to each target gene were developed in our laboratory with Thermo Scientific[TM] InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, USA) to use as positive controls for each reaction.

Each reaction consisted of 2 µl 10x PCR buffer, 2 µl of a 2.5 mM deoxyribonucleotide, 0.2 µl itaq[TM] plus DNA polymerase enzyme (iNtRON Biotechnology, Korea), 0.5 µl of each forward and reverse primer (Pacific Science, Thailand), DNA template 20 ng, sterile deionized water up to total volume of 20 µl. Sequences of the primer sets for the studied genes were described at Table 1[7,12]. The amplification of polymerase chain reaction was performed in Veriti 96 well Thermal Cycler system (Applied Biosystems, USA). The cyclic conditions included initial denaturation of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, with a final extension step at 72°C for 7 min. The amplified products were resolved by electrophoresis in 1.8% agarose gels in Tris-acetate-EDTA buffer (TAE). Digital images of the RedSafe™ (iNtRON Biotechnology, Korea) nucleic acid stained gels were obtained with the ChemiDoc™ MP imaging system (Bio-Rad, USA).

Data analysis was conducted using a statistical package program SPSS (SPSS Inc, Chicago, USA). Odds ratio of clinical periodontal parameters and Pg was calculated. Clinical periodontal data were compared among healthy control and periodontitis groups with positive or

negative sites of various Pg genotypes using Kruskal-Wallis test. Correlation between the presence of different Pg genotypes and clinical parameters was assessed with Spearman's correlation test. Significant level was considered at p<0.05.

Primer	Sequence (5' to 3')	Amplicon size (bp)
fimA genotype II- Forward	GCATGATGGTACTCCTTTGA	292
fimA genotype II- Reverse	CTGACCAACGAGAACCCACT	
16S rRNA gene of Pg- Forward	TGTAGATGACTGATGTGTAAAACC	197
16S rRNA gene of Pg- Reverse	ACGTCATCCCCACCTTCCTC	

Table 1. Primers for universal of all Pg strains and fimA type 2 specific genotype used in the conventional PCR assay.

Results

The current study involved 28 subjects with equal number of males and females at age from 20 to 74 years old. Samples were isolated from 42 periodontal pocket sites at depth 4 to 9 mm and from 7 healthy periodontium sites at depth 1-3 mm. In periodontitis sites, 81% samples were positive for Pg while the occurrence of Pg was not detected in healthy controls. The association between the presence of Pg and clinical periodontal status were presented in table 2. Increased odds of having CAL and deep pockets were found to relate with the presence of Pg (p<0.05).

Parameter	Odds ratio	95%CI	p-value
Loss of attachment (CAL $\geq$ 3 mm)	14.000	2.428-80.731	0.002
Loss of attachment (CAL$\geq$5mm)	8.938	2.222-35.953	0.002
Deep Pocket (PD $\geq$ 6 mm)	13.591	2.602-70.982	0.001
Bleeding on probing	3.600	1.012-12.811	0.045
Gingival index	2.095	0.610-7.200	0.192
Amount of subgingival calculus	6.563	1.532-28.120	0.012

Table 2. Odds ratio of clinical periodontal parameters with presence of Pg.
*Fisher's Exact test, *p*<0.05

In periodontitis samples, the distribution of Pg strains according to fimA genotypes was found with PgfimAII positive (42.9%, n=18), Pg other genotypes positive as shown by 16S rRNA gene positive and fimA genotype 2 negative (38.1%, n=16), and Pg detection negative (19.0%, n=7). Clinical periodontal status among groups were demonstrated in Table 3. Kruskal-Wallis test revealed the difference of PD level and CAL between the PgfimAII positive group and Pg negative groups from both healthy and periodontitis sites (p<0.05). The correlation

between the presence or absence of different Pg genotypes and clinical periodontal parameters were displayed in Table 4. Periodontitis sites of all groups showed moderate to strong correlation with CAL, PD, and calculus amount according to calculus index score. The correlation levels of BOP and gingival index were weak with slight difference among groups.

	Healthy	Periodontitis		
	Pg negative (n=7)	Pg negative (n=8)	Pg other genotypes positive (n=16)	PgFimAII positive (n=18)
CAL (mm)	0.14±0.38	4.75±1.28*	5.75±2.54*	7.00±2.35*#
PD (mm)	2.00±0.58	5.00±1.07*	6.13±1.67*	6.56±1.50*#
BOP (%)	28.57	50.00	75.00	66.67
Gingival index	0.86±0.69	1.75±0.71	1.69±0.60	1.83±0.71
Calculus index	1.00±0.82	1.88±0.64	1.94±0.57*	2.28±0.58*

Table 3. Comparison of average value, standard deviation and percentage of clinical parameters among periodontitis and healthy groups according to Pg genotype categories.

Parameter	PgFimAII positive r-value (p-value)	Other Pg strains positive r-value (p-value)	Pg negative r-value (p-value)
CAL	0.892 (0.000)	0.766 (0.000)	0.932 (0.000)
PD	0.854 (0.000)	0.854 (0.000)	0.947 (0.000)
Severe CAL ($\geq$ 5 mm)	0.764 (0.000)	0.633 (0.001)	0.564 (0.029)
Deep PD ($\geq$ 6 mm)	0.649 (0.000)	0.580 (0.004)	0.367 (0.179)
BOP	0.345 (0.092)	0.438 (0.037)	0.218 (0.435)
Gingival index	0.402 (0.046)	0.364 (0.088)	0.406 (0.134)
Calculus index	0.653 (0.000)	0.549 (0.007)	0.533 (0.041)

*Spearman's Correlation test, p<0.05

Table 4. Correlation between presence of different Pg genotypes and clinical periodontal characteristics in periodontitis sites.

Discussion

Progression of periodontitis was reflected by destructive change of clinical periodontal status. The clinical manifestations that represented severe stage of periodontal destruction consisted of $\geq$ 5-mm CAL with > 6-mm PD at gingival inflamed area with BOP which required complex management[3]. Calculus amount especially at subgingival area impacted the periodontitis progression as its porous structure and surface roughness could harbor high proportion of anaerobic bacteria which resulted in retaining of biofilms and bacterial toxins[14-15]. The influence of specific bacterium on periodontal diseases could be evaluated through the correlation with the representative clinical parameters as demonstrated by previous studies with other bacterial species such as Treponema Lecithinolyticum or Tannerella Forsythia[16-17].

Pg has been implicated as a key pathogen for periodontitis owing to its own pathogenic capacity and its synergistic role for other periodontal pathogenic bacteria in plaque biofilms complex[18-19]. The odds result from Table 2 provided confirmation regarding the infectious role of Pg in periodontitis with strong relationship to deep pocket formation and CAL. The association of Pg with periodontitis from this study was comparable to reports from Japan (odds ratio 11.788)[20] and China (odds ratio 16.36)[13] but was lower than data from Korean population (odds ratio 25.18)[12]. The difference from these findings might be described by varied sampling sites, periodontal conditions, and ethnicity[21].

Pathogenicity of Pg has been relevant to multiple virulent factors such as fimbriae, lipopolysaccharide, proteinases and cysteine proteases[6,22-23]. The function of major fimbriae at cell surface of Pg resulted in colonization at tooth surfaces which promoted invasion into host tissues and negative stimulation of host responses[6,24]. Diversities of fimA genotypes affected morphology of fimbriae in Pg which was suggested their attributes of different virulence[8-9]. Various distribution of fimA-specific *Pg* genotypes in periodontal pockets may indicate the risk of periodontal destruction as represented by distinctive periodontal characteristics in Table 3. CAL and PD levels from PgfimAII group was significantly higher than Pg negative group but the difference was not found when compared with Pg possessing other fimA genotypes. PgfimAII has been recognized as the first periodontitis-related strain followed by Pg possessing type 4 and type 1b genotypes[7-8,10,12,25].

The findings from Table 4 indicated that discrepancies of fimA genotypes had impact on clinical periodontitis indicators. Stronger correlations with deep PD and severe CAL were noticed in PgfimAII group. The other fimA genotypes displayed considerable correlation to periodontal destruction which may be the outcome, in parts, from other virulent genotypes. Further studies to evaluate the clinical role of each Pg genotype and other specific periodontal pathogens could possibly clarify our understanding in etiological factors of periodontal diseases.

Conclusions

Pocket formation, CAL and calculus amount are parameters that involved with periodontitis sites containing any Pg genotypes or other periodontal pathogens. Severe CAL and deep PD strongly associated with PgfimAII positive sites with different levels from Pg negative sites. Heterogeneity of Pg genotypes did not relate strongly to BOP or gingival index.

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Declaration of Interest

The authors report no conflict of interest.

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