Association of Gene Expression of Porphyromonas Gingivalis with Cigarette Smoking and Periodontal Pocket Depth

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Abstract

Periodontal disease is a complex disease that involves many bacteria including Porphyromonas gingivalis (P. gingivalis) in its pathophysiological process. We sought to analyze and correlate levels of P. gingivalis based on smoking status and periodontal pocket depth in periodontitis subjects.

This was a cross-sectional study of smokers aged 35 to 60 years with periodontitis. Clinical data (periodontal pocket depth), smoking status information (pack-years), and Gingival Crevicular Fluid (GCF) samples were collected. The gene expression of P. gingivalis in GCF was measured by quantitative real-time polymerase chain reaction (qPCR). Spearman correlation testing between P. gingivalis in GCF and periodontal pocket depth and smoking status was completed.

P. gingivalis findings in shallow pocket versus deep pocket populations (P=0.72) and nonsmokers, moderate smokers, and heavy smokers (P=0.258) showed no significant difference. No significant correlation was present between P. gingivalis proportion and periodontal pocket depth (r=0.128; P=0.552), while the correlation between P. gingivalis and smoking status was moderate but statistically insignificant (r=0.488; P=0.016).

Periodontal pocket depth and smoking status are not primary factors influencing the periodontal microflora, although variable gene expression of P. gingivalis was observed between groups.

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Introduction

While periodontology has experienced a number of advancements in the past few decades, the treatment of periodontal disease still requires significant attention. In the United States, the incidence of periodontal disease is about 42%, with 7.8% being patients with severe periodontitis.[1] According to the Ministry of Health of Indonesia, in 2011, the prevalence of periodontal disease was 60% in the total Indonesian population, being the second most common oral disease nationwide.[2]

Periodontal disease is generally caused by subgingival and supragingival plaque accumulation, which leads to calculus, and gingival inflammation. Plaque accumulation facilitating the growth of bacterial colonies at the dentogingival region border is the main factor causing periodontal disease, both in cases of gingivitis, and in those of periodontitis.[3] Periodontal disease is a complex disease involving many bacteria in its pathophysiological process. One of the common bacteria found in chronic periodontitis is *Porphyromonas gingivalis (P. gingivalis),* acting as a key periodontal pathogen.[4,5] The stages of lesion formation due to inflammation in periodontal disease can be stratified as initial lesion, early lesion, established lesion, and advanced lesion. The difference between gingivitis and periodontitis is that gingivitis does not cause damage to connective tissue and bone, while periodontitis is destructive to ligaments and bones, potentially causing tooth loss.[3,6]

In periodontitis, several risk factors are known to have an impact on the healing process of patients.[7,8] Local predisposing factors that can affect periodontitis include occlusal trauma and microbiome changes due to smoking habits.[9]

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Basic Health Research (Riskesdas) conducted in 2013 by the Ministry of Health of Indonesia stated that 24.3% of the total Indonesian population smokes and this number continues to increase in 2018. Based on the history of cigarettes smoked, which is calculated as the number of pack-years of cigarettes smoked (pack-year = number of packs per day × number of years smoking), smokers can be classified into the following five groups: nonsmokers, very light/occasional smokers (> 0–5.2 pack-years), moderate smokers (5.3–15 pack-years), and heavy smokers (> 15 pack-years).[10]

The habit of consuming tobacco causes a buildup of nicotine and cotinine in the oral cavity and is known to promote environmental changes. Nicotine and its metabolites can facilitate the formation of anaerobic conditions, with the potential to change the normal microbiota balance.[11,12] Smoking can also inhibit the neutrophil apoptosis process, which is considered to contribute to the inflammatory process and destruction of periodontal tissue.

A study by Adler et al. found that smokers had significantly deeper periodontal pockets relative to nonsmokers,[13] while an in vitro investigation by Hanioka et al. determined that cigarette smoke extract upregulates *P. gingivalis* major fimbrial antigen and promotes the colonization and infection of *P. gingivalis*, although the role of nicotine is not directly related to this growth. Furthermore, the study stated that cigarette smoke extract not only alters *P. gingivalis'* gene expression and outer membrane proteins but also the host's humoral responses against *P. gingivalis*, increasing their infectivity. Further research is still needed to explain the role of smoking or nicotine in *P. gingivalis* growth. In this study, we sought to find an association between cigarette smoking exposure in Indonesian subjects and the proportion of *P. gingivalis* growth as one of the key periodontal pathogens.

Materials and methods

The Ethical Committee of the Faculty of Dentistry at Universitas Indonesia approved this study.

This study was performed by residents in periodontology at Universitas Indonesia. Written informed consent was obtained from each patient prior to their involvement. The study subjects were patients who came to a private clinic in Bekasi and were diagnosed with periodontitis. Maximal pocket depth (PD) was used as the primary indicator for periodontitis.[14] The inclusion criteria were 35 to 60 years of age, smoker, diagnosed with periodontitis (PD ≥ 4 mm), no scaling in the last six months, and willingness to be a research sample and sign the informed consent form. The exclusion criteria were pregnancy; use of steroids, anticonvulsants, or anticoagulants; and the presence of systemic diseases such as diabetes mellitus and immune deficiency syndrome.

A total of 30 individuals participated in this study. All subjects were asked to fill out a questionnaire that included questions about smoking habits and Gingival Crevicular Fluid (GCF) samples were taken to analyze the *P. gingivalis* bacterial proportion. Periodontal PD measurements were performed at six sites (i.e., buccal-mesial, mid-buccal, buccal-distal, palatal/lingual-mesial, mid-palatal/lingual, and palatal/lingual-distal) of each tooth. The GCF samples were taken from the deepest pocket in all quadrants. Before taking the sample, the removal of supragingival plaque and isolation of the site from saliva were completed. A #30 sterile paper point was inserted gently into the periodontal pocket with a PD of 4 mm or more and left for 30 seconds. Samples that were contaminated with blood were not included. The paper points that were obtained from every patient were placed into Eppendorf tubes containing 1 mL of TE buffer and then were placed in a cool box and immediately taken to the laboratory to be stored at −80°C. The GCF samples were processed in the Oral Biology Laboratory of the Faculty of Dentistry, Universitas Indonesia using quantitative polymerase chain reaction (qPCR) to analyze the bacterial proportion.

First, dioxyribonucleic acid (DNA) was extracted using Trizol from all samples, then the DNA samples were quantitated using the *Qubit*® dsDNA *HS* (High-sensitivity) Assay Kit. All samples were run in duplicate in MicroAmp™ Fast Optical 48-well plates in the Applied Biosystems StepOne Plus system (Applied Biosystems, Foster City, CA, USA). The protocol was as follows: 95°C for three minutes, followed

by 40 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Following the amplification, melting curve analysis was completed to verify the authenticity of the amplified products by their specific melting temperatures using Polymerase chain reaction (Applied Biosystems). PCR amplification was carried out in 10-µL reactions containing 3 µL of DNA, 0.5 µL of each specific primer (1 µL), 5 µL of *SYBR* Green, and 1 µL of nuclease-free water. Primer sequences of 16sRNA as the housekeeping gene were: forward 5'-TGTAGATGAC TGATGGTGAAA-3' and reverse 5'-ACTGTTAGCAACTACCGATGT-3'. Primer sequences of *P. gingivalis* were: forward 5'-TACCCATCGTCGCCTTGGT-3' and reverse 5'-CGGACTAAAACCGCATACACTTG-3'.

The collected data for *P. gingivalis* proportion were compared between shallow periodontal pockets and deep periodontal pockets using the Mann–Whitney U test, while those for *P. gingivalis* proportion between smoking groups were compared using the Kruskal–Wallis test. Correlations between periodontal PD and smoking status with *P. gingivalis* proportion in GCF were measured using Spearman's rank correlation. A p-value of less than 0.05 was considered to be significant. All data were analyzed statistically using the Statistical Package for the Social Sciences version 25.0 (IBM Corp., Armonk, NY, USA).

Results

Considering measurements from each respondent, 15 had periodontal PDs of 4 to 5 mm, categorized into the shallow pocket group, and 15 had periodontal PDs of 6 mm or more, categorized into the deep pocket group (Table 1). The respondents were also grouped based on their smoking status: 12 respondents were nonsmokers, six respondents were moderate smokers (> 15 but ≤ 30 pack-years), and 12 respondents were heavy smokers (> 30 pack-years).

From these six groups, the six respondents in the group of nonsmokers with shallow PDs became the control samples. Given this fact, proportion of *P. gingivalis* in the shallow pocket category was 8% higher for moderate smokers and 54% higher for heavy smokers. Meanwhile, *P. gingivalis* proportion in the deep pocket category was 27% higher for nonsmokers, 42% higher for moderate smokers, and 154% higher for heavy smokers (Figure 1).

Demographic characteristics	
Age (years)	44.13 (7.36)
Sex	
Male	20 (66.67%)
Female	10 (33.33%)
Smoking status	
Nonsmoker	12 (40%)
Moderate smoker	6 (20%)
Heavy smoker	12 (40%)

Table 1. Demographic data.

*Smoking status was measured using the pack-year formula.[10]

Light smoker: > 5.2 and ≤ 15 pack-years; moderate smokers: > 15 and ≤ 30; and heavy smokers: > 30 pack-years.

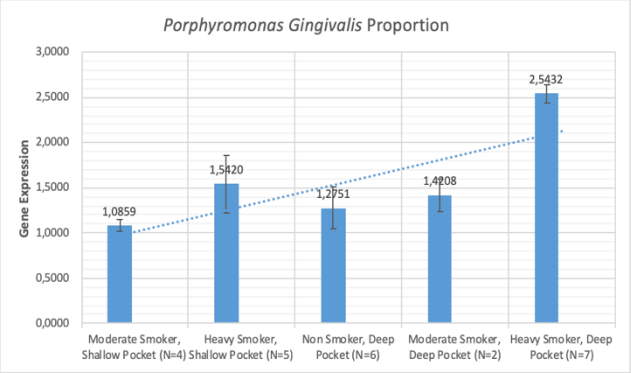

Figure 1. Analysis of *P. gingivalis* proportion with periodontal PD (pocket depth) and smoking status using qPCR (quantitative real-time polymerase chain reaction).

* Nonsmoker, shallow pocket (n=6) was used as baseline.

Pocket Depth	*P. gingivalis* Median (min–max)	p*P*-value
Shallow pocket	0.0035 (0.0010–0.0614)	0.726
Deep pocket	0.0042 (0.0006–0.1432)	

Table 2. Comparative analysis of *P. gingivalis* among smokers based on periodontal PD.

Mann–Whitney U test; * *P*<0.05, significant difference

Table 2 shows a comparison between *P. gingivalis* proportion in the GCF of shallow PDs and deep PDs. The Mann–Whitney U test shows a p-value of 0.7267 (*P*≥0.05).

Smoking Status	*P. gingivalis* Median (min–max)	p*P*-value
NonNonsmoker	0.0035 (0.0006–0.1432)	0.258
Moderate smoker	0.0026 (0.0010–0.0614)	
Heavy smoker	0.0082 (0.0013–0.0250)	

Table 3. Comparative analysis of *P. gingivalis* among smokers based on smoking status.

Kruskal–Wallis test; * *P*<0.05, significant difference

Table 3 shows a comparison between *P. gingivalis* proportion in GCF based on smoking status. The Kruskal–Wallis test shows a p-value of 0.258 (*P*≥0.05).

Parameter	*P. gingivalis* R (*P*-value)
Periodontal pocket depth	0.128 (0.552)
Smoking status	0.488 (0.016)*

Table 4. Correlation analysis between periodontal PD and smoking status with *P. gingivalis.*

Spearman correlation test; * *P*<0.05, significant difference

The correlations between periodontal PD and smoking status relative to *P. gingivalis* proportion in GCF are shown in Table 4. A positive correlation was found between periodontal pocket depth and *P. gingivalis* proportion in GCF but the finding was statistically not significant and the correlation was also weak. A positive correlation between *P. gingivalis* proportion in GCF and smoking status was found to be moderate and statistically significant.

Discussion

In this study, *P. gingivalis* was found more often in deep periodontal pockets than in shallow periodontal pockets, but the difference was not significant (Table 2). This result is in contrast with research by Klein et al. and Kulkarni et al., who showed that *P. gingivalis* was found more frequently in patients with PDs of greater than 5 mm rather than 5 mm or less, and the difference in such was significant.[15,16] Kulkarni et al. also found a statistically significant positive correlation existed between PD and the presence of *P. gingivalis*. Elsewhere, a study by Orzechowka et al. showed that, in pockets of 5 mm and pockets of 7 mm or more, the occurrence of *P. gingivalis* was significantly more frequent among patients with chronic periodontitis. However, there was no significant correlation between the prevalence of *P. gingivalis* and periodontal PD.[17]

This study also determined that *P. gingivalis* was found more often in heavy smokers than moderate and nonsmokers (Table 3), similar to as seen in studies by Bagaitkar et al. and Zeller et al. that reported an increased prevalence of *P. gingivalis* was found among smokers relative to nonsmokers; conversely, other research showed that no statistically significant difference in *P. gingivalis* prevalence was present between smokers and nonsmokers.[18–20]

Interestingly, this study indicates smoking status has more correlation to *P. gingivalis* proportion than periodontal PD in that a higher concentration of *P. gingivalis* was found in heavy smokers with shallow pockets than in nonsmokers and moderate smokers with deep pockets [Table 4]. Bagaitkar et al. found that cigarette smoking increases one's vulnerability to *P. gingivalis* infection and susceptibility to periodontitis while, at the same time, reducing clinical signs of inflammation. It has been hypothesized that cigarette smoke suppresses the host's immunity and contributes to an increase in bacterial virulence.[21]

Several studies have previously implicated tobacco smoke in the increasing virulence of *P. gingivalis.* George et al. investigated the mechanism of cigarette smoke promoting genomic evolution in *P. gingivalis* and found that smokers are more prone to infection with *P. gingivalis* and to developing periodontitis, yet exhibit reduced clinical inflammation. These authors' experimental results provided further evidence suggesting cigarette smoke to be an important factor in bacterial evolution, which may explain in part the effects that tobacco smoke has in altering the virulence of this key periodontal pathogen that may contribute to the emergence of more virulent strains.[22] Shah et al. suggested that smoking affects the periodontium primarily by interfering with the body response mechanisms rather than by adding to local destructive factors. Nicotine affects the

fibroblasts by increasing the production of collagen but impairs collagen secretion. It also changes the cell structure of the fibroblasts, making them more susceptible to periodontal damage.[23]

Epithelial cells are the first line of defense against bacterial infection. Although the exact cigarette smoke mechanism acting to affect cellular functions remains unknown, a study by Bondy-Carey et al. showed that cigarette smoke extract (CSE) exposure promoted *P. gingivalis* survival and invasion by reducing the proinflammatory cytokine burden.[24] Research by Shin and Lee stated that CSE had the potential to elicit a stress reaction and so may serve as an environmental modulating factor for *P. gingivalis* growth and survival.[25] *P. gingivalis* adapts CSE by altering the expression of several virulence factors, including major and minor fimbrial antigens (FimA and Mfa1, respectively). FimA has been shown to play a critical role in *P. gingivalis* colonization of the periodontium through strong interactions with several host proteins, including collagen, laminin, and fibronectin, and by promoting adherence to the oral epithelia and to other plaque bacteria such as *Streptococcus* spp.[21]

In this study, we did not analyze factors such as bleeding index or oral hygiene index of the patient. A study by Christopher et al. showed that the prevalence and level of *P. gingivalis* are low in groups with good oral hygiene.[26]

Separately, Condorelli et al. found that *P. gingivalis* was isolated more from active sites than inactive sites of periodontal patients.[27] The presence of bleeding on probing at isolated sites is not a particularly good indicator of active inflammation, but the absence of bleeding on probing is a good indicator of periodontal stability.[28] Therefore, further studies with larger sample sizes and that assess more confounding factors are needed.

Conclusions

This present study revealed that *P. gingivalis* in GCF was higher in periodontitis subjects with deep pockets relative to shallow pockets, but the difference was not significant. Also, *P. gingivalis* in GCF was higher in periodontitis subjects with a heavy smoking status than in those with a moderate smoking status or nonsmokers. This study indicates that *P. gingivalis* proportion is more affected by smoking status rather than periodontal PD.

Further studies are needed to give us a better understanding of how much of a key driving force smoking status is in altering the virulence of *P. gingivalis,* which may infect smokers, and nonsmokers alike.

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Declaration of Interest

There are no conflicts of interest to disclose.

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