Prognostic Role of Porphyromonas Gingivalis in Oral Cavity Squamous Cell Carcinoma Patients

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Abstract

Background: Oral microbial species play direct and/or indirect role in carcinogenesis of oral cavity squamous cell carcinoma (OSCC). Porphyromonas gingivalis (Pg) has been identified a correlation with OSCC. Fimbriae play a vital role for its attribution of initial attachment and adhesion of Pg. Six genotypes (types I-V, Ib) of fimA were identified based on sequence variations and the genotype was suggested a relationship to pathogenicity of Pg.

Objective: To investigate the abundance of Pg in OSCC as well as the frequency of Pg fimA genotypes in OSCC patients.

Methods: Ninety-five OSCC patients and thirty-nine gender- and age-matched non-OSCC subjects were investigated abundance of Pg in saliva. Presence of Pg was compared in OSCC tissue and para-cancerous tissue from patient as well. Clinical data were extracted and patients followed up for a mean period of 13 months. Presence of Pg and fimA genotypes were investigated in OSCC tissue and in saliva, then PCR products were sequencing and compared.

Results: OSCC patients showed high abundance of Pg in saliva (Chi-square=14.531, P=0.001). OSCC tissue showed strong in situ expression of Pg by in situ hybridization compared with normal tissue adjacent to OSCC. Patients with overabundance of Pg in saliva are associated with systemic disease (Chi-square=10.328, P=0.029), longer disease-free time (Z=-2.988, P=0.003), and lower recurrence rate (Chi-square=5.670, P=0.017). The abundance of Pg was an independent favorable prognostic factor (HR: 0.124, 95%CI: 0.016 to 0.941). There was dominant distribution of Pg with genotype I+Ib (21.1%), II (31.6%) and IV (21.1%) in OSCC patients. The fimA genotypes detected in saliva were in accordance with those in OSCC tissue, and there was significantly correlation in amplified Pg fragments similarity between in saliva and in OSCC tissue.

Conclusions: This study indicated that Pg might involve in the pathogenesis of OSCC, and Pg might consider as a potential prognostic indicator in OSCC. There was a dominant distribution of Pg with genotypes I, Ib, II and IV in OSCC patients. The presence of Pg in tumor might be saliva in provenance.

Background

There were approximately 354,864 new cases of lip and oral cavity cancer and 177,384 deaths from these tumors worldwide in 2018[1]. Oral cavity squamous cell carcinoma (OSCC) is the most common malignant disease in head and neck besides non melanoma skin cancer. Traditionally, risk factors associated with OSCC include tobacco and alcohol consumption, betel quid chewing, HPV or HIV infection, dietary factor, vitamin and mineral deficiencies, occupational exposures and heritable conditions. But overwhelming epidemiologic, clinicopathologic and molecular studies have proved oral microbial species play direct and/or indirect role in carcinogenesis of OSCC[2].
Porphyromonas gingivalis (Pg), an anaerobic Gram-negative bacterium, has been suggested that correlates to OSCC in extensively studies\cite{3-9}. But the abundance of Pg capable of predicting the recurrence as a biomarker still unknown. Therefore, it is urgent to stratify OSCC patients based on evaluation of the abundance of Pg due to the risk of misinformation and misconception. Furthermore, fimbriae play a vital role for its attribution of initial attachment and adhesion, although Pg has a number of virulence factors, such as gingipain, lipopolysaccharide, capsule and fimbriae\cite{10}. The major fimbriae fimA and the minor one mfa1 were two distinct fimbriae expressed in Pg.

Six genotypes (types I-V, Ib) of fimA were identified based on sequence variations and the genotype was suggested a relationship to pathogenicity of Pg\cite{11, 12}. Previous studies have reported a high prevalence of fimA genotype II in periodontitis patients, whereas others shown that fimA genotype Ib, II and IV are more aggressive\cite{13, 14}.

The abundance of Pg for predicting recurrence outcomes and the distribution of fimA genotypes in cancer is yet to be elucidated. In this study, we explored the association between Pg and OSCC, and its potential prognostic value in OSCC was also assessed. Meanwhile, we investigated the prevalence of fimA genotypes in OSCC at last.

**Methods**

Approval from the institutional review board was obtained at the Hospital of Stomatology Wuhan University before starting the study (2016-60). Informed consent was obtained from each patient.

The inclusion criterion was patients with primary OSCC. The exclusion criteria were patients: 1) received oral prophylaxis in the latest three months, 2) undergone radiotherapy and/or chemotherapy before surgery, 3) edentulous, 4) refused to receive surgery, 5) disagreed to participate in this study.

Between October 2018 and April 2019, 95 patients with OSCC (65 male and 30 female subjects, aged 21-82 years, mean age 55.8) treated in hospital were included in this study. TNM classification was stratified in accordance with the eighth edition of the American Joint Committee on Cancer\cite{15}. Except for one patient with bone metastatic OSCC, all patients were M0 stage. The control group comprised 39 OSCC-free subjects (21 males and 18 females, aged 33-76 years, mean age 52.6) diagnosed with salivary gland disease, lymphadenopathy, lymphoma, buccal or tongue chronic infection, epulis, ranula, lipoma, lymphoepithelial cyst and sebaceous gland carcinoma.

Clinical records were retrieved and excisional specimens were available during the operation. Assessed clinicopathological variables included age, gender, systematic disease, location of tumor, size, pathological report, smoking, alcoholic consumption, body mass index (BMI) and treatment. Systemic disease included hypertension, diabetes, coronary arteries arteriosclerosis and chronic hepatitis B. All patients underwent complete resection, surgery + radiotherapy (IMRT: 76 Gray to 63 Gray) or surgery +
chemoradiotherapy (Docetaxel, Cisplatin, 5-Fluorouracil) respectively. Pathological diagnosis was established by one pathologist and confirmed by another experienced pathologist.

Patients were followed up from discharge by telephone or clinical assessment. Pathologic confirmation of recurrence was obtained in patients with clinical signs or symptoms. Disease-free survival (DFS) is defined as the time (in months) from the date of discharge to April 2020 or until the date recurrence was diagnosed.

A total of 134 saliva samples were collected between 6 a.m. and 8 a.m. following an overnight fast and refrainment of tooth brushing. Subjects were asked to swish vigorously with 40 mL sterilised double distilled water (bacteria negative in PCR assay) for 1 minute, and then to expectorate in to another specimen tube[16]. The saliva samples were centrifuged at 14,000 rpm for 15 minutes, and then the cell pellet was suspended in 1 mL of sterile TE buffer. Saliva samples stored at -80 ºC until testing.

Bacterial DNA was extracted from saliva samples using a commercial DNA extraction kit (DP302, Tiangen, China) according to the manufacturer’s protocol, except adding an enzymatic lysis step with lysozyme (20 mg/ml, 37 ºC, 60 minutes). The resultant DNA was stored at -20 ºC until in PCR.

A total of 15 out of 95 OSCC patients fresh-frozen OSCC tissue samples were obtained during the operation. DNA was extracted using the Total DNA/RNA/Protein Kit (R6734, Omega Bio-tek, USA) according to the procedure recommended by the manufacturer. Quantification of \(Pg\) in saliva samples and detection of \(fimA\) genotypes were measured by Real time quantitative PCR. Amplifications were performed in duplicated on Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, USA). The primers, synthesized by Sangon Biotech (Shanghai, China), used in Real time quantitative PCR with the annealing temperature is listed in Table 1.

Table 1. Specific oligonucleotides used in this study.
| Primer         | Sequence (5’-3’)                  | Annealing temperature (°C) | Reference |
|---------------|-----------------------------------|-----------------------------|-----------|
| Universal primers | F: TCCTACGGGAGGCAGCAGT            | 60                          | [17]      |
|               | R: GGACTACCAGGGTATCTAATCCTGTT     |                             |           |
| *Pg*          | F: ACCTTACCGGGATTGAAATG           | 58                          | [17]      |
|               | R: CAACCATGCAGCACCTACATAGAA       |                             |           |
| *fimA I*      | F: CTGTGTGTGTGGGCAAACCTTC         | 58                          | [18]      |
|               | R: AACCCCGCTCCCTGTATTCCGA         |                             |           |
| *fimA Ib*     | F: CAGCAGAGCCAAAAAACAATCG         | 58                          | [11]      |
|               | R: TGTCAAGATAATTAGCGTCTG          |                             |           |
| *fimA II*     | F: GCATGATGGTACTCCCTTTGA          | 58                          | [19]      |
|               | R: CTGACCACCGAGAACCCACT           |                             |           |
| *fimA III*    | F: ATTACACCTACACAGGTGAGGC         | 58                          | [18]      |
|               | R: AACCCCGCTCCCTGTATTCCGA         |                             |           |
| *fimA IV*     | F: CTTACGTTGCTATTACCCAA           | 58                          | [18]      |
|               | R: AACCCCGCTCCCTGTATTCCGA         |                             |           |
| *fimA V*      | F: ACAACAGTCTCCTTGAGCT           | 58                          | [11]      |
|               | R: TATGGGAGGCTGAAGGTACTG          |                             |           |
| *Pg* probe used in ISH | CAATACTCGTATCGGCCGTTACC-Digoxin |                             | [3]       |

*Pg*: *Porphyromonas gingivalis*. ISH: In situ hybridization.

The reaction mixture of 20 μL was composed of 50 ng saliva DNA template or 2 μL tissue DNA template, 0.4 μM of the specific primer, ChamQTM SYBR® qPCR Master with a final concentration of 1X (Q311, Vazyme, China) and appropriate dose of sterilized DNase-RNase-free water. The conditions for Real time quantitative PCR were as follows: 94 °C for 5 minutes, then 28 cycles for *Pg* or 40 cycles for *fimA* genotypes of 30 s at 94 °C, 45s at 58 °C or 60 °C, and 1 min at 72 °C; with a final extension of 10 min at 72 °C. Melting curves were generated from 60 °C to 95 °C and read every 0.5 °C for 5 seconds. An average Ct value was obtained. The Ct for *Pg* was determined by subtracting the Ct value of *Pg* from that of universal primer. The relative abundance of *Pg* was calculated by the 2^ΔΔCt method.

Amplified PCR products of *fimA* genotype from Real time quantitative PCR were checked on 2% agarose gel (ST004L, Beyotime, China). This was done using 1X Tris Acetate-EDTA buffer (TAE) from 50X TAE
Gels were stained with 4S GelRed (A616697, Sangon Biotech, China). Image results were captured with the digital imaging system (NuGenius, SYNGENE, UK). One pair of amplified \textit{Pg} fragments from OSCC tissue and saliva were confirmed following nucleotide sequencing by Sangon Biotech (Shanghai, China) and the correlation of two sequence by aligning two sequences with BLAST (http://www.ncbi.nlm.nih.gov/BLAST)\cite{20}.

Among 15 OSCC tissue patients, remaining OSCC tissue and normal tissue adjacent to OSCC from one patient were fixed in 4\% paraformaldehyde, paraffin-embedded, and cut into 4 \textmu m sections, which was stained with haematoxylin and eosin, gram and subjected to in situ hybridization (ISH) using Enhanced Sensitive ISH Detection kit I (POD) (MK1030, Boster, China) according to the manufacturer's instructions. The probe is listed in Table 1. Omission of the probe was obtained as the negative controls.

**Statistical analysis**

Shapiro–Wilk test was used to assess whether or not data were normally distributed. Normally distributed data were analysed by Student's t test and presented as Mean ± Standard Deviation. The data without normal distribution presented as median and inter-quartile range (M, Q) and analysed by the Mann-Whitney U test. Categorical variables were analysed by Pearson Chi-square test or Fisher's exact test. The cutoff point to convert the number of \textit{Pg} 16S rRNA gene copies into categorical data (weak, <2.04 and strong, >2.04) was performed using X-tile software\cite{21}. The Kaplan–Meier method was used to estimate the recurrence rate. Univariate and multivariate Cox regression models were used to evaluate the association between DFS and clinicopathological variables. Parameters considered statistically significant ($P<0.20$) in the univariate model were analysed in the multivariate models with backward selection. All two-tailed $P$ values $<$ 0.05 were considered as significant. All analyses were carried out using IBM SPSS Statistics software (IBM SPSS Statistics V.25.0, USA).

**Results**

*Overabundance of \textit{Pg} was associated with the incidence of OSCC*

As showed in table 2, compared with controls matched for gender and age ($P<0.05$), OSCC patients showed overabundance of \textit{Pg} in saliva ($P<0.05$). To exclude contamination of samples, \textit{Pg} was also detected in tissues by ISH from one patient. As showed in figure 1, compared with normal tissue which is adjacent to OSCC, OSCC tissue showed strong in situ expression of \textit{Pg}.

Table 2. Overabundance of \textit{Pg} in saliva from OSCC patient.
|                  | Weak | Strong | Chi-square | P     |
|------------------|------|--------|------------|-------|
| OSCC             | 67   | 28     | 14.531     | 0.001 |
| OSCC-free        | 39   | 0      |            |       |

The abundance of *P. gingivalis* was a favorable independent prognostic factor

Ninety-four patients were available for the follow-up visit, and one patient was non-responsive to any form of contact. The deadline for follow-up was April 2020. After a median follow-up period of 13 months (range 3–17 months), recurrence was diagnosed as end point for seventeen patients, with a 18.1% (17/94) cumulative recurrence rate (figure 2).

Clinicopathological information of OSCC patients is shown in table 3. Distribution of clinicopathological outcomes was compared with the abundance of *P. gingivalis* to assess the potential prognostic variables. Overabundance of *P. gingivalis* in saliva was more likely to correspond with systemic disease (*Chi-square*=10.328, *P*=0.029). Compared with weak group, patients with the overabundance of *P. gingivalis* in saliva had a longer disease-free time (*Z*=-2.988, *P*=0.003). Patients with the overabundance of *P. gingivalis* in saliva had a lower recurrence rate than those with low abundance of *P. gingivalis* (*Chi-square*=5.670, *P*=0.017). However, differences were not statistically significant by age, gender, T stage, N stage, M stage, BMI, smoking, alcoholic consumption, location, differentiation grade, treatment and *fimA* genotypes. Neither single *fimA* genotype was statistically significant associated with any clinicopathological parameters.

Table 3. Clinicopathological details.
| Parameters                        | Number (%) | Porphyromonas gingivalis | \( P \) value |
|----------------------------------|------------|--------------------------|---------------|
|                                  |            | Weak | Strong                  |               |
| Age (years)                      | 55.8±12.7  | 95 (100) | 57.0±11.9 | 53.0±14.4 | 0.197 |
| Gender                           |            |      |                          |               |
| Male                             | 65 (68.4)  | 43   | 22                       | 0.169 |
| Female                           | 30 (31.6)  | 24   | 6                        |       |
| Systemic disease                 |            |      |                          |               |
| No                               | 69 (72.6)  | 53   | 16                       | 0.029 |
| Yes                              | 26 (27.4)  | 14   | 12                       |       |
| T stage                          |            |      |                          |               |
| T1+T2                            | 87 (91.6)  | 63   | 24                       | 0.229 |
| T3+T4                            | 8 (8.4)    | 4    | 4                        |       |
| N stage                          |            |      |                          |               |
| N0                               | 74 (77.9)  | 54   | 20                       | 0.326 |
| N1+N2                            | 21 (22.1)  | 13   | 8                        |       |
| M stage                          |            |      |                          |               |
| M0                               | 94 (98.9)  | 66   | 28                       | 0.999 |
| M1                               | 1 (1.1)    | 1    | 0                        |       |
| Smoking                          |            |      |                          |               |
| No                               | 45 (47.4)  | 32   | 13                       | 0.906 |
| Yes                              | 50 (52.6)  | 35   | 15                       |       |
| Alcoholic consumption            |            |      |                          |               |
| No                               | 57 (60.0)  | 40   | 17                       | 0.927 |
| Yes                              | 38 (40.0)  | 27   | 11                       |       |
| Body mass index                  | 23.5, 4.4  | 88 (92.6) | 23.2, 4.1 | 24.5, 3.8 | 0.062 |
| Location                         |            |      |                          |               |
| Buccal                           | 21 (24.2)  | 19   | 4                        | 0.595 |
| Tongue                           | 52 (54.7)  | 34   | 18                       |       |
| Gingiva                          | 11 (11.6)  | 7    | 4                        |       |
| Floor of mouth                   | 5 (5.3)    | 4    | 1                        |       |
| Hard palate                      | 4 (4.2)    | 3    | 1                        |       |
| Differentiation grade            |            |      |                          |               |
| Well                             | 17 (19.1)  | 11   | 6                        | 0.565 |
| Medium                           | 65 (73.0)  | 48   | 17                       |       |
| Poor                             | 7 (7.9)    | 5    | 2                        |       |
| FimA genotypes                   |            |      |                          |               |
| I+Ib                             | 20 (21.1)  | 13   | 7                        | 0.903* |
| II                               | 30 (31.6)  | 22   | 8                        |       |
| Stage | Count | Median M | M+R | Total |
|-------|--------|----------|-----|-------|
| III   | 4 (4.2)| 3        | 1   |       |
| IV    | 20 (21.1)| 15     | 5   |       |
| V     | 2 (2.1)| 1        | 1   |       |
| I+Ib+II | 1 (1.1) |        |     |       |
| I+Ib+III | 2 (2.1) |        |     |       |
| I+Ib+IV | 1 (1.1) |        |     |       |
| I+Ib+V | 1 (1.1) |        |     |       |
| II+IV | 3 (3.2) |          |     |       |
| I+Ib+II+IV | 1 (1.1) |        |     |       |
| Untyped | 10 (10.5) |     |     |       |

| Treatment† | Count | Median M | M+R | Total |
|------------|--------|----------|-----|-------|
| Surgery    | 64 (68.1)| 47     | 17  | 0.606 |
| Surgery + Radiotherapy | 22 (23.4)| 14     | 8   |       |
| Surgery + Chemoradiotherapy | 8 (8.5) | 5      | 3   |       |

| Outcome†   | Count | Median M | M+R | Total |
|------------|--------|----------|-----|-------|
| Recurrence | 17 (18.1)| 16    | 1   | 0.017 |
| Disease-free | 77 (81.9)| 50 | 27 |       |

| Disease-free time (months) | Count | Median M | M+R | Total |
|---------------------------|--------|----------|-----|-------|
| 13, 3                    | 94 (98.9)| 13, 2  | 14, 3| 0.003 |

*: Kruskal-Wallis H test was used among fimA genotype I+Ib, II, III, IV, V.
†: One patient dropped out in follow-up.

Univariate analysis showed that M stage, smoking, the abundance of Pg and BMI were trended with prognosis (P< 0.2). To adjust for those variables, the parameters listed in table 4 were calculated in a multivariate analysis through a backward elimination process. Statistically, the abundance of Pg was an independent favorable prognostic factor (P=0.043), while age, gender, FimA genotypes, Systemic disease, TNM stage, smoking, alcoholic consumption, location, BMI, differentiation grade and treatment were not.

Table 4. Univariate and multivariate variable Cox regression model analysis of parameters for time to recurrence
### Parameters

| Parameters       | Univariate |           |           | Multivariate |           |           |
|------------------|------------|-----------|-----------|--------------|-----------|-----------|
|                  |            | HR        | 95%CI     | P Value      | HR        | 95%CI     | P Value |
| Age              |            | 1.021     | 0.981 to 1.063 | 0.306        | -          | -         | -       |
| Gender           |            | 1.492     | 0.568 to 3.920 | 0.417        | -          | -         | -       |
| FimA genotypes   |            | 0.999     | 0.963 to 1.036 | 0.946        | -          | -         | -       |
| Systemic disease |            | 1.068     | 0.376 to 3.037 | 0.902        | -          | -         | -       |
| T stage          |            | 1.335     | 0.304 to 5.856 | 0.702        | -          | -         | -       |
| N stage          |            | 1.471     | 0.518 to 4.175 | 0.469        | -          | -         | -       |
| M stage          |            | 8.861     | 1.134 to 69.226 | 0.038        | -          | -         | -       |
| Smoking          |            | 0.485     | 0.179 to 1.313 | 0.155        | -          | -         | -       |
| Alcoholic consumption |        | 1.474     | 0.569 to 3.822 | 0.425        | -          | -         | -       |
| Location         |            | 1.050     | 0.713 to 1.548 | 0.805        | -          | -         | -       |
| Body mass index  |            | 0.858     | 0.719 to 1.024 | 0.090        | -          | -         | -       |
| Differentiation grade |      | 1.304     | 0.507 to 3.350 | 0.582        | -          | -         | -       |
| Treatment        |            | 1.273     | 0.646 to 2.508 | 0.485        | -          | -         | -       |
| Pg               |            | 0.135     | 0.018 to 1.018 | 0.052        | 0.124      | 0.016 to 0.941 | 0.043 |

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**Dominant distribution of Pg with genotype I, Ib, II and IV in OSCC patients**

Amplified PCR products of fimA genotype were checked on 2% agarose gel electrophoresis. The distribution of fimA genotype from saliva of 95 OSCC patients was listed in Table 3. FimA genotype I and Ib was detected in 20 (21.1%) specimens, genotype II in 30 (31.6%) specimens, genotype III in 4 (4.2%) specimens, genotype IV in 20 (21.1%) specimens, genotype V in 2 (2.1%) specimens. We also found two or more genotypes of fimA from one sample. FimA genotype I, Ib and II was detected in 1 (1.1%) participant, genotype I, Ib and III in 2 (2.1%) participants, genotype I, Ib and IV in 1 (1.1%) participant, genotype I, Ib and V in 1 (1.1%) participant, genotype II and IV in 3 (3.2%) participants, genotype I, Ib, II and IV in 1 (1.1%) participant. Ten participants showed negative on 2% agarose gel electrophoresis assay. This finding supported the dominant distribution of Pg with genotype I, Ib, II and IV in saliva from OSCC patients.

**The presence of Pg in tumor were saliva in provenance**

To clarify the homogeneity of Pg among saliva and OSCC tissue, the frequency of fimA genotypes was also detected in fifteen OSCC tissues. Among fifteen patients, the fimA genotypes detected in saliva were in accordance with those in OSCC tissue (Table 5). Besides, amplified Pg fragments from OSCC tissue
and saliva were examined in one patient, we found significantly correlation in nucleotide similarity (Figure 3 and Figure 4). Collectively, these results provided evidence supporting the presence of \( Pg \) in tumor was saliva in provenance, and the profile of \( Pg \) in saliva was in accordance with those in tissue.

Table 5. The frequency of \( fimA \) genotypes in saliva and in oral squamous cell carcinoma tissues.

| I+Ib | II | III | IV | V | I+Ib+III |
|------|----|-----|----|---|----------|
| Saliva | 3 | 6 | 1 | 2 | 2 | 1 |
| Tissue | 3 | 6 | 1 | 2 | 2 | 1 |

**Discussions**

With recent breakthroughs in high-throughput genetic-based tools, there has been a hot issue concerning the relationship between the oral microbiome and neoplasms, especially OSCC. Recently accumulating evidence indicated the relationship between \( Pg \) and OSCC. Immortalized oral keratinocytes stimulated with \( Pg \) led to a more aggressive malignant profile phenotype and contributing to enhanced tumor features\[22\]. The serum immunoglobulin G antibody against \( Pg \) was higher in OSCC patients compared with non-OSCC patients\[4\]. \( Pg \) increased the size and the multiplicity of carcinoma to promote the development of oral cancer\[5\]. Previous studies have confirmed the association between \( Pg \) and OSCC by examining the abundance of \( Pg \) in saliva of patients, and unveiled that patients with medium and poor differentiation, overall clinical stage III and stage IV as well as lymph node metastasis suggested association with \( Pg \) involvement\[3\]. Our study also verified the overabundance of \( Pg \) from OSCC patients compared with non-OSCC subjects in saliva. To exclude contamination of samples, we examined the presence of \( Pg \) in OSCC tissue by ISH. There was high enrichment of \( Pg \) in OSCC tissue compared with normal tissue adjacent to OSCC, as previous study\[3\]. After an average follow-up period of 13 months, the disease recurred in 18.1% (17/94) of our patients. As the same as our findings, it was reported early stage patients have a 90-95% survival rate for one year or more, and advanced stage patients have a 65-70% survival rate\[23\].

We found that overabundance of \( Pg \) in saliva was more likely to correspond with systemic disease. Systemic disease included hypertension, diabetes, coronary arteries arteriosclerosis and chronic hepatitis B. It is may because diabetes mellitus and hypertension were correlation with increasing abundance of \( Pg \)\[24, 25\].

Compared with weak group, patients with the overabundance of \( Pg \) in saliva had longer disease-free time, and patients with the overabundance of \( Pg \) in saliva had a lower recurrence rate than those with low abundance of \( Pg \). Those suggested that \( Pg \) may have an effect on prognosis of oral carcinoma. Unexpectedly, we found the overabundance of \( Pg \) is a prognostic factor for longer DFS. Contrary to popular belief, they found that \( Pg \) was associated with higher risk of pancreatic cancer\[26, 27\], esophageal squamous cell carcinoma\[28, 29\] and oral squamous cell carcinoma\[3\]. Meanwhile, \( Pg \) was associated with
overall survival rate in esophageal squamous cell carcinoma\textsuperscript{[30]}. Furthermore, patients with high level of \textit{Pg} had the worst prognosis in esophageal squamous cell carcinoma\textsuperscript{[29]}. Besides population and follow-up period contributed to this prognostic incongruity, the inherent mechanistic also needs to elucidate.

One of the most vital virulence of \textit{Pg} has been supposed to the presence of fimbriae, which plays an important role in adhesion, colonization and invasion to tissues\textsuperscript{[31]}. Most of studies focused on distribution of \textit{fimA} genotypes in periodontitis. However, the frequency of \textit{fimA} genotype of \textit{Pg} in OSCC was not clear. \textit{FimA} genotypes I and Ib could be discriminated by Rsa I enzyme digest, however, clearly discrimination of genotypes I and Ib seem to be improbable\textsuperscript{[11]}. Besides, there were no differences in immunological analysis between \textit{fimA} I and Ib fimbriae\textsuperscript{[11]}. So we consider \textit{fimA} genotypes I and Ib as a whole.

In this study, the association of \textit{fimA} genotypes and clinicopathological parameters was not statistically significant. However, the predominant detected \textit{fimA} genotypes in OSCC were genotypes I, Ib, II and IV. Several studies concluded that nucleotide genetic variation was likely associated with virulence. Some reported \textit{fimA} genotypes Ib, II and IV are the most virulent fimbriae in periodontitis and assist in adhesion and invasion\textsuperscript{[11, 32]}. It was reported that \textit{fimA} genotype Ib, II and IV led to more severe infections and inflammations\textsuperscript{[33, 34]}. Clinical isolation of \textit{Pg} from chronic periodontitis patients also supported the virulence of \textit{fimA} genotypes Ib, II and IV\textsuperscript{[35]}. Different \textit{Pg fimA} genotypes was injected subcutaneously and Nakano et al found that the weakest inflammatory response was induced by genotype III\textsuperscript{[34]}. \textit{FimA} genotype V was the least amount of genotypes in this study. The reason might be the low prevalence (0%-29\%) of this genotype reported in other studies\textsuperscript{[36]}. Single \textit{Pg fimA} genotype was determined more than 70\% of OSCC patients, and two or more genotypes were also detected in a subset of the subjects. Approximately 10\% of the samples were multiple genotypes in this population, which was less than the results of other studies\textsuperscript{[11, 14]}. Researchers attributed to the limitations of PCR in discrimination of \textit{fimA} genotypes and the possibility of classifying new genotypes\textsuperscript{[37]}. Due to conservative properties of DNA, the bacterial 16S ribosomal DNA allows identification of the genus and species. Analysis of \textit{Pg} nucleotide sequences in the OSCC tissue and in the saliva showed a homology of 100\%. Moreover, the distribution of \textit{fimA} genotypes in OSCC tissue is according to those in saliva. Those results support the provenance of \textit{Pg} in OSCC tissues is those in saliva\textsuperscript{[38]}. The limitation of this study is the short follow-up period. Further research will need to be done to elucidate the prognostic role of \textit{Pg} in the long run. Except fimbriae of \textit{Pg}, other important virulence such as: encapsulation (K1-K6), gingipain (types A, B, C) as well as lysine-specific types I and II, may also play a vital role in the association between \textit{Pg} and OSCC. Those remain to be uncovered in the future study.

Conclusions
This study found the overabundance of *Pg* was associated with OSCC. However, patients with systemic disease, longer disease-free time and the lower recurrence rate were related to the overabundance of *Pg*. Meanwhile, the abundance of *Pg* was an independent favorable prognostic factor. Furthermore, there was a dominant distribution of *Pg* with genotype I, Ib, II and IV from OSCC patients and the presence of *Pg* in tumor was saliva in provenance.

**Abbreviations**

Oral cavity squamous cell carcinoma: OSCC  
*Porphyromonas gingivalis*: *Pg*  
In situ hybridization: ISH

**Declarations**

**Ethics approval and consent to participate**

Approval from the institutional review board was obtained at the Hospital of Stomatology Wuhan University before starting the study (2016-60). Informed consent was obtained from each patient. Animal Ethics clearance is not applicable, as this study does not involve any animals.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analysed during this study are included in this published article.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

QC, Z Shao, Z Shang conceived the study; QC, KL performed the experiments; XZ, LW performed the statistical analysis; QC and Z Shao wrote the initial draft of the manuscript; LT, EJ revised and edited the manuscript; Z Shang finalised the manuscript; All authors read and approved the final manuscript.
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Figures

Figure 1

Expressions of Porphyromonas gingivalis in oral cavity squamous cell carcinoma (OSCC) (A), normal tissue adjacent to OSCC (B) and probe-free as negative control (D) by in situ hybridization; Gram staining (C).
Figure 2

Cumulative recurrence curve of 94 patients.
Figure 3

The PCR product examined of *Porphyromonas gingivalis* in saliva (A) and in oral cavity squamous cell carcinoma tissue (B).

| Score       | Expect | Identities | Gaps  | Strand |
|-------------|--------|------------|-------|--------|
| 154 bits(170) | 2e-43  | 85/85(100%) | 0/85(0%) | Plus/Plus |

Query 1: TCAACCATGCACTACCTACTAGACGCCCCTAAAGGGAGTGCAACATCTCTGATCTGGGT 60

Sbjct 1: TCAACCATGCACTACCTACTAGACGCCCCTAAAGGGAGTGCAACATCTCTGATCTGGGT 60

Query 61: CTGCAATTCACCCG6GTAAGGGTA 85

Sbjct 61: CTGCAATTCACCCG6GTAAGGGTA 85

Figure 4

The homology analysis of *Porphyromonas gingivalis* detected in saliva and oral cavity squamous cell carcinoma tissue.