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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[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[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*[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[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[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 arteriesarteriosclerosis 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: ACCTTACCCGGGATTGAAATG	58	[17]
	R: CAACCATGCAGCACCTACATAGAA		
fimA I	F: CTGTGTGTTTATGGCAAACTTC	58	[18]
	R: AACCCCGCTCCCTGTATTCCGA		
fimA Ib	F: CAGCAGAGCCAAAAACAATCG	58	[11]
	R: TGTCAGATAATTAGCGTCTGC		
fimA II	F: GCATGATGGTACTCCTTTGA	58	[19]
	R: CTGACCAACGAGAACCCACT		
fimA III	F: ATTACACCTACACAGGTGAGGC	58	[18]
	R: AACCCCGCTCCCTGTATTCCGA		
fimA IV	F: CTATTCAGGTGCTATTACCCAA	58	[18]
	R: AACCCCGCTCCCTGTATTCCGA		
fimA V	F: AACAACAGTCTCCTTGACAGTG	58	[11]
	R: TATTGGGGGTCGAACGTTACTGTC		
Pg probe used in ISH	CAATACTCGTATCGCCCGTTATTC-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 ºC for 5 minutes, then 28 cycles for *Pg* or 40 cycles for *fimA* genotypes of 30 s at 94 ºC, 45s at 58 ºC or 60 ºC, and 1 min at 72 ºC; with a final extension of 10 min at 72 ºC. Melting curves were generated from 60 ºC to 95 ºC and read every 0.5 ºC for 5 seconds. An average Ct value was obtained. The ΔCt 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 $2^{-\Delta\Delta Ct}$ 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

(ST716, Beyotime, China). Gels were stained with 4S GelRed (A616697, Sangon Biotech, China). Image results were captured with the digital imaging system (NuGenius, SYNGENE, UK). One paire of amplified *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)[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 μm 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 *Pg* 16S rRNA gene copies into categorical data (weak, <2.04 and strong, >2.04) was performed using X-tile software[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 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 *Pg* in saliva (P < 0.05). To exclude contamination of samples, *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 *Pg*.

Table 2. Overabundance of *Pg* in saliva from OSCC patient.

	Porphyromonas gingivalis			
	Weak	Strong	*Chi-square*	*P*
OSCC	67	28	14.531	0.001
OSCC-free	39	0		

The abundance of Pg 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 *Pg* to assess the potential prognostic variables. Overabundance of *Pg* 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 *Pg* in saliva had a longer disease-free time (*Z*=-2.988, *P*=0.003). Patients with the overabundance of *Pg* in saliva had a lower recurrence rate than those with low abundance of *Pg* (*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	

	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†	Surgery	64 (68.1)	47	17	0.606
	Surgery + Radiotherapy	22 (23.4)	14	8	
	Surgery + Chemoradiotherapy	8 (8.5)	5	3	
Outcome†	Recurrence	17 (18.1)	16	1	0.017
	Disease-free	77 (81.9)	50	27	
Disease-free time (months)	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	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

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 arteriesarteriosclerosis 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[30]. Furthermore, patients with high level of *Pg* had the worst prognosis in esophageal squamous cell carcinoma[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 *Pg* has been supposed to the presence of fimbriae, which plays an important role in adhesion, colonization and invasion to tissues[31]. Most of studies focused on distribution of *fimA* genotypes in periodontitis. However, the frequency of *fimA* genotype of *Pg* in OSCC was not clear. *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[11]. Besides, there were no differences in immunological analysis between *fimA* I and Ib fimbriae[11]. So we consider *fimA* genotypes I and Ib as a whole.

In this study, the association of *fimA* genotypes and clinicopathological parameters was not statistically significant. However, the predominant detected *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 *fimA* genotypes Ib, II and IV are the most virulent fimbriae in periodontitis and assist in adhesion and invasion[11, 32]. It was reported that *fimA* genotype Ib, II and IV led to more severe infections and inflammations[33, 34]. Clinical isolation of *Pg* from chronic periodontitis patients also supported the virulence of *fimA* genotypes Ib, II and IV[35]. Different *PgfimA* genotypes was injected subcutaneously and Nakano et al found that the weakest inflammatory response was induced by genotype III[34]. *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[36]. Single *PgfimA* 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[11, 14]. Researchers attributed to the limitations of PCR in discrimination of *fimA* genotypes and the possibility of classifying new genotypes[37].

Due to conservative properties of DNA, the bacterial 16S ribosomal DNA allows identification of the genus and species. Analysis of *Pg* nucleotide sequences in the OSCC tissue and in the saliva showed a homology of 100%. Moreover, the distribution of *fimA* genotypes in OSCC tissue is according to those in saliva. Those results support the provenance of *Pg* in OSCC tissues is those in saliva[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 *Pg* in the long run. Except fimbriae of *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 *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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References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018, 68(6):394-424.

[2] Karpiński TM. Role of Oral Microbiota in Cancer Development. Microorganisms. 2019, 7(1):E20.

[3] Chang C, Geng F, Shi X, Li Y, Zhang X, Zhao X, et al. The prevalence rate of periodontal pathogens and its association with oral squamous cell carcinoma. Appl Microbiol Biotechnol. 2019, 103(3):1393-1404.

[4] Park DG, Woo BH, Lee BJ, Yoon S, Cho Y, Kim YD, et al. Serum Levels of Interleukin-6 and Titers of Antibodies Against *Porphyromonas gingivalis* Could Be Potential Biomarkers for the Diagnosis of Oral Squamous Cell Carcinoma. Int J Mol Sci. 2019, 20(11):E2749.

[5] Wu JS, Zheng M, Zhang M, Pang X, Li L, Wang SS, et al. *Porphyromonas gingivalis* Promotes 4-Nitroquinoline-1-Oxide-Induced Oral Carcinogenesis With an Alteration of Fatty Acid Metabolism. Front Microbiol. 2018, 9:2081.

[6] Utispan K, Pugdee K, Koontongkaew S. Porphyromonas gingivalis, lipopolysaccharide-induced macrophages modulate proliferation and invasion of head and neck cancer cell lines. Biomed Pharmacother. 2018, 101:988-995.

[7] Groeger S, Jarzina F, Domann E, Meyle J. Porphyromonas gingivalisactivates NFκB and MAPK pathways in human oral epithelial cells. BMC Immunol. 2017, 18(1):1.

[8] Woo BH, Kim DJ, Choi JI, Kim SJ, Park BS, Song JM, et al. Oral cancer cells sustainedly infected with Porphyromonas gingivalis exhibit resistance to Taxol and have higher metastatic potentia. Oncotarget, 2017, 8(29):46981-46992.

[9] Ha NH, Woo BH, Kim DJ, Ha ES, Choi JI, Kim SJ, et al. Prolonged and repetitive exposure to Porphyromonas gingivalis increases aggressiveness of oral cancer cells by promoting acquisition of cancer stem cell properties. Tumour Biol. 2015, 36(12):9947-9960.

[10] Abreu MGL, Kawamoto D, Mayer MPA, Pascoal VDB, Caiaffa KS, Zuza EP, et al. Frequency of Porphyromonas gingivalis fimA in smokers and nonsmokers after periodontal therapy. J Appl Oral Sci. 2019, 27:e20180205.

[11] Nakagawa I, Amano A, Ohara-Nemoto Y, Endoh N, Morisaki I, Kimura S, et al. Identification of a new variant of fimA gene of Porphyromonas gingivalis and its distribution in adults and disabled populations with periodontitis. J Periodontal Res. 2002, 37(6):425-432.

[12] Nagano K, Hasegawa Y, Abiko Y, Yoshida Y, Murakami Y, Yoshimura F. Porphyromonas gingivalis FimA fimbriae: fimbrial assembly by fimA alone in the fim gene cluster and differential antigenicity among fimA genotypes. PLoS One, 2012, 7(9):e43722.

[13] Miura M, Hamachi T, Fujise O, Maeda K. The prevalence and pathogenic differences of Porphyromonas gingivalis fimA genotypes in patients with aggressive periodontitis. J Periodontal Res. 2010, 40(2):147-152.

[14] Amano A, Kuboniwa M, Nakagawa I, Akiyama S, Morisaki I, Hamada S. Prevalence of specific genotypes of Porphyromonas gingivalis fimA and periodontal health status. J Dent Res. 2000, 79(9):1664-1668.

[15] MB Amin, SB Edge, FL Greene, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2017.

[16] Fan X, Peters BA, Min D, Ahn J, Hayes RB. Comparison of the oral microbiome in mouthwash and whole saliva samples. PLoS One, 2018, 13(4):e0194729.

[17] Al-Rawi N, Al-Marzooq F. The Relation between Periodontopathogenic Bacterial Levels and Resistin in the Saliva of Obese Type 2 Diabetic Patients. J Diabetes Res. 2017, 2017:2643079.

[18] Amano A, Nakagawa I, Kataoka K, Morisaki I, Hamada S. Distribution of Porphyromonas gingivalis strains with fimA genotypes in periodontitis patients. J Clin Microbiol. 1999, 37(5):1426-1430.

[19] Moon JH, Shin SI, Chung JH, Lee SW, Amano A, Lee JY. Development and evaluation of new primers for PCR-based identification of type II fimA of Porphyromonas gingivalis. FEMS Immunol Med Microbiol. 2012, 64(3):425-428.

[20] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990, 215(3):403-410.

[21] Camp RL, Dolled-Filhart M, Rimm DL. X-Tile: A New Bio-Informatics Tool for Biomarker Assessment and Outcome-Based Cut-Point Optimization[J]. Clin Cancer Res. 2004, 10(21):7252-7259.

[22] Hoppe T, Kraus D, Probstmeier R, et al. Stimulation with Porphyromonas gingivalis enhances malignancy and initiates anoikis resistance in immortalized oral keratinocytes. J Cell Physiol. 2019,234(12):21903-21914.

[23] Chaturvedi P, Singh A, Chien CY, et al. Tobacco related oral cancer[J]. BMJ. 2019,5(365): l2142.

[24] Gogeneni H, Buduneli N, Ceyhan-Öztürk B, et al . Increased infection with key periodontal pathogens during gestational diabetes mellitus[J]. J Clin Periodontol. 2015,42(6):506-512.

[25] Hanaoka Y, Soejima H, Yasuda O, et al. Level of serum antibody against a periodontal pathogen is associated with atherosclerosis and hypertension[J]. Hypertens Res. 2013,36(9):829-833.

[26] Fan X, Alekseyenko AV, Wu J, et al. Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case-control study[J]. Gut. 2018,67(1):120-127.

[27] Michaud DS, Izard J, Wilhelm-Benartzi CS, et al. Plasma antibodies to oral bacteria and risk of pancreatic cancer in a large European prospective cohort study. Gut[J]. 2013,62(12):1764-1770.

[28] Peters BA, Wu J, Pei Z, et al. Oral Microbiome Composition Reflects Prospective Risk for Esophageal Cancers[J]. Cancer Res. 2017,77(23):6777-6787.

[29] Gao SG, Yang JQ, Ma ZK, et al. Preoperative serum immunoglobulin G and A antibodies to Porphyromonas gingivalis are potential serum biomarkers for the diagnosis and prognosis of esophageal squamous cell carcinoma[J]. BMC Cancer. 2018,18(1):17.

[30] Gao S, Li S, Ma Z, et al. Presence of Porphyromonas gingivalis in esophagus and its association with the clinicopathological characteristics and survival in patients with esophageal cancer[J]. Infect Agent Cancer. 2016,11:3.

[31] Lin X, Wu J, Xie H. Porphyromonas gingivalis minor fimbriae are required for cell-cell interactions. Infect Immun. 2006, 74(10):6011-6015.

[32] Missailidis CG, Umeda JE, Ota-Tsuzuki C, Anzai D, Mayer MP. Distribution of fimA genotypes of Porphyromonas gingivalis in subjects with various periodontal conditions. Oral Microbiol Immunol, 2004, 19(4):224-229.

[33] Amano A, Nakagawa I, Okahashi N, Hamada N. Variations of Porphyromonas gingivalis fimbriae in relation to microbial pathogenesis. J Periodontal Res. 2004, 39(2):136-142.

[34] Nakano K, Kuboniwa M, Nakagawa I, Yamamura T, Nomura R, Okahashi N, et al. Comparison of inflammatory changes caused by Porphyromonas gingivalis with distinct fimA, genotypes in a mouse abscess model. Oral Microbiol Immunol. 2004, 19(3):205-209.

[35] Enersen M, Olsen I, Kvalheim Ø, Caugant DA. fimA genotypes and multilocus sequence types of Porphyromonas gingivalis from patients with periodontitis. J Clin Microbiol. 2008, 46(1):31-42.

[36] Moreno S, Jaramillo A, Parra B, Botero JE, Contreras A. Porphyromonas gingivalis Fim-A genotype distribution among Colombians. Colomb Med (Cali). 2015, 46(3):122-127.

[37] Perez-Chaparro PJ, Rouillon A, Minet J, Lafaurie GI, Bonnaure-Mallet M. fimA genotypes and PFGE profile patterns in Porphyromonas gingivalis isolates from subjects with periodontitis. Oral Microbiol Immunol. 2009, 24(5):423-426.

[38] Castillo-Rojas G, Cerbón MA, López-Vidal Y. Presence of Helicobacter pylori in a Mexican Pre-Columbian Mummy. BMC Microbiol. 2008, 8(1):119.

Figures

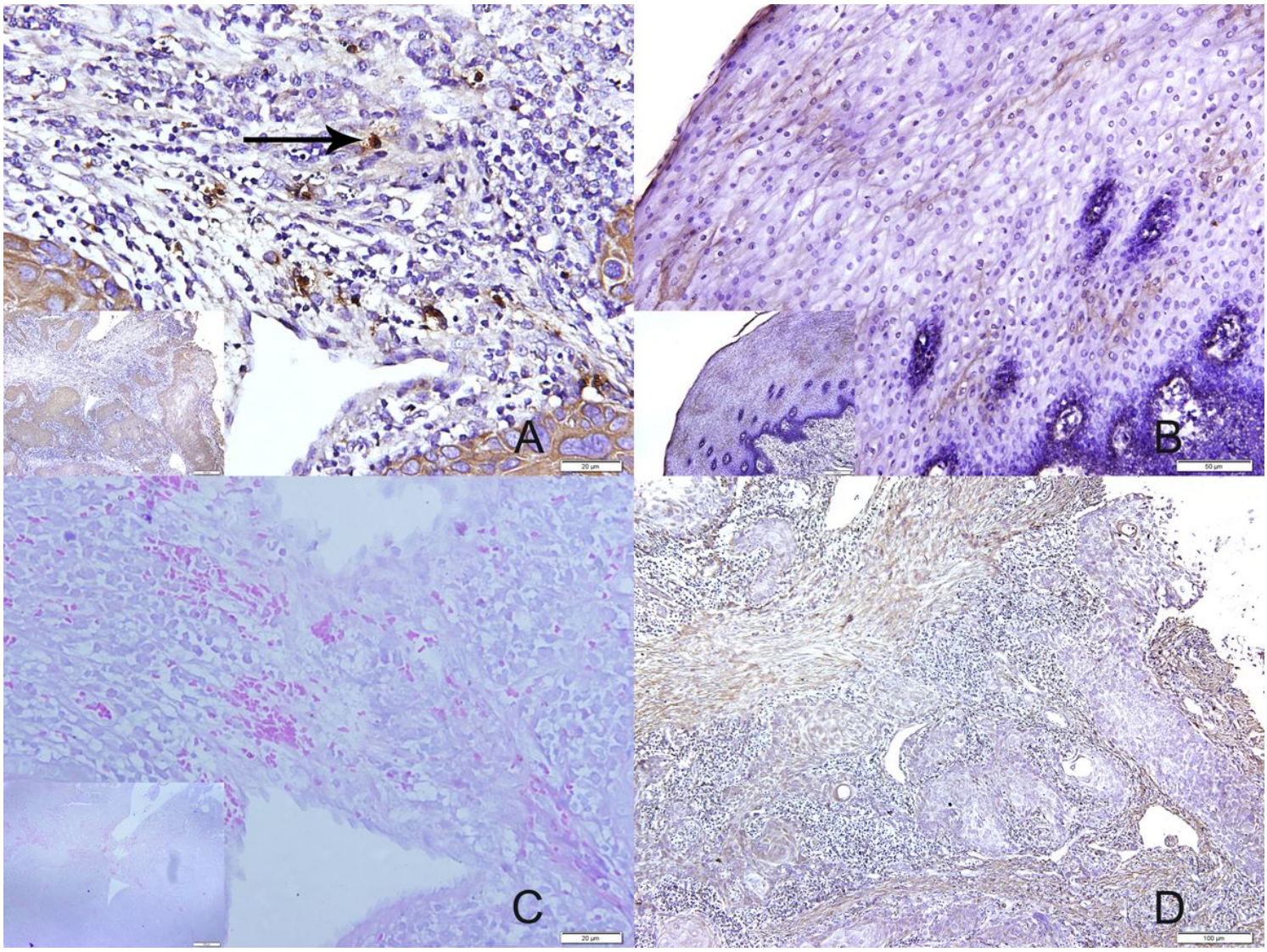

Figure 1

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).

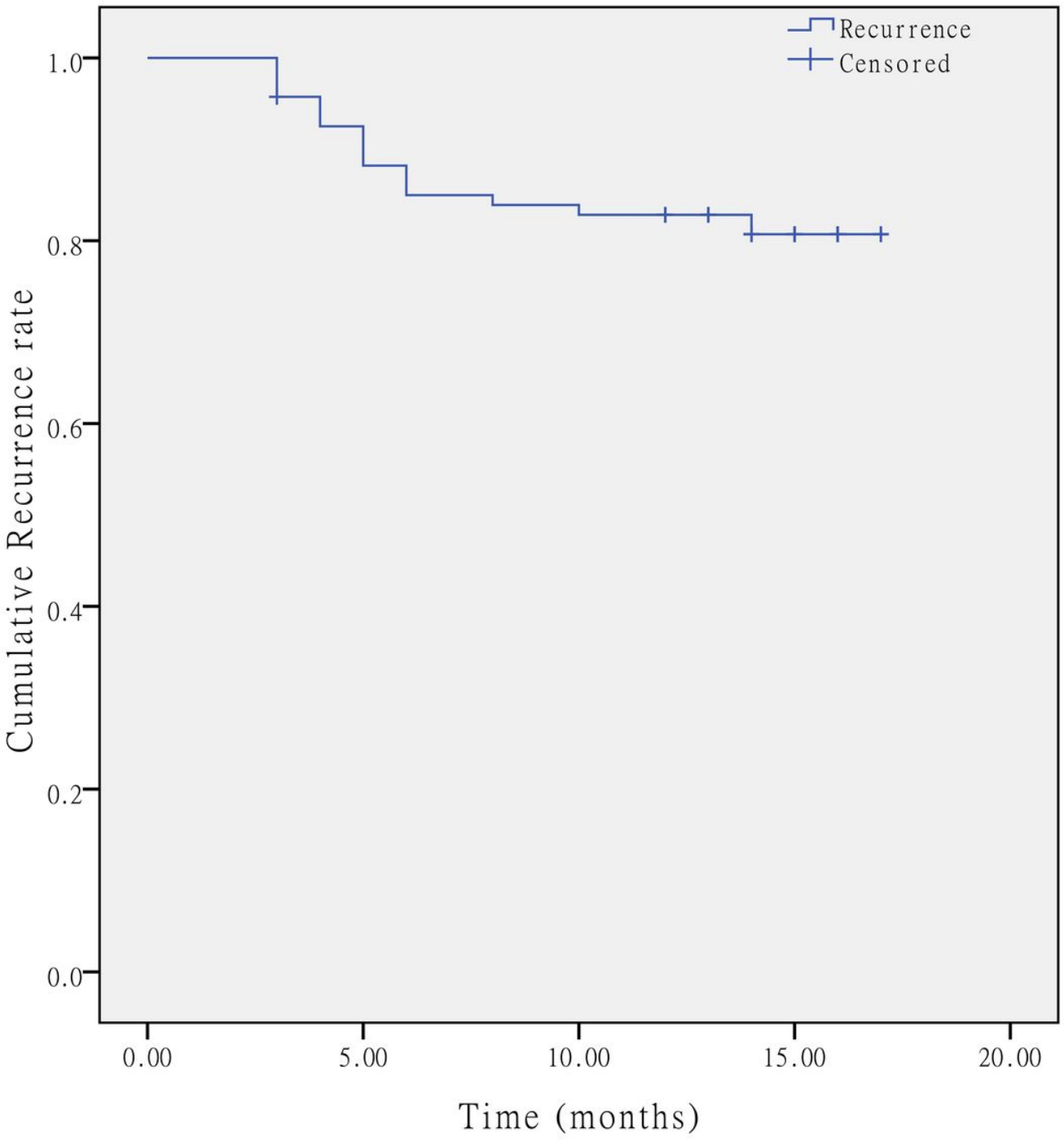

Figure 2

Cumulative recurrence curve of 94 patients.

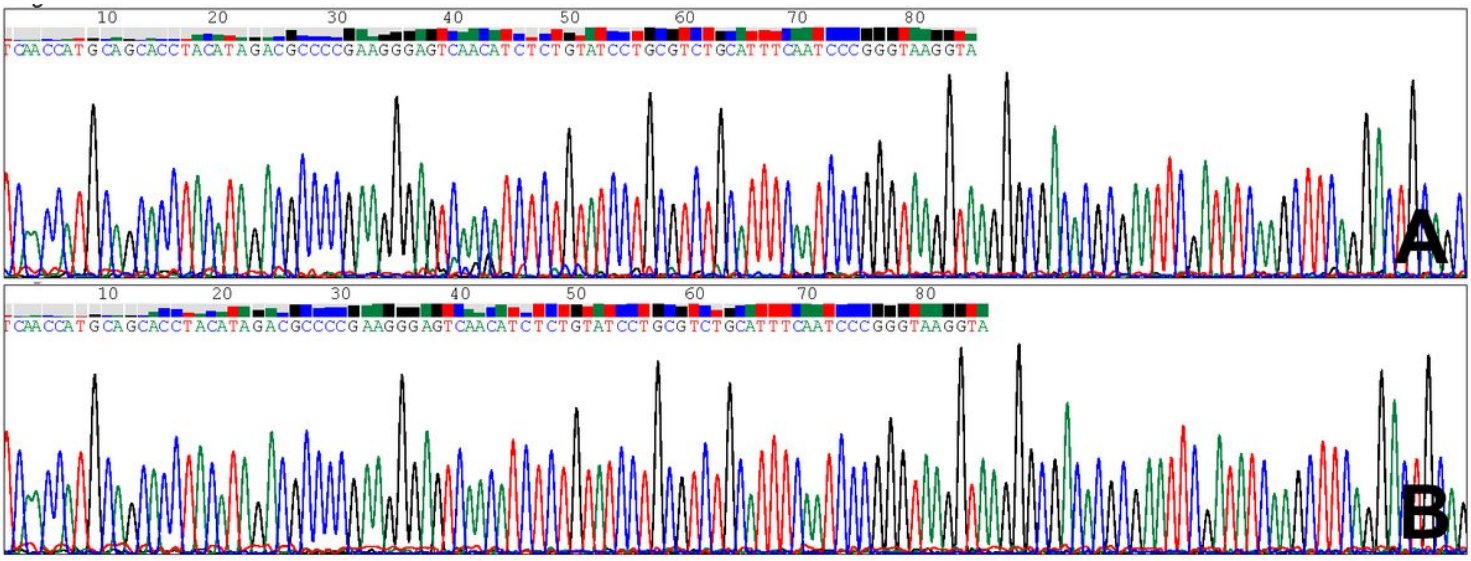

Figure 3

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   TCAACCATGCAGCACCTACATAGACGCCCCGAAGGGAGTCAACATCTCTGTATCCTGCGT  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1   TCAACCATGCAGCACCTACATAGACGCCCCGAAGGGAGTCAACATCTCTGTATCCTGCGT  60

Query  61  CTGCATTTCAATCCCGGGTAAGGTA  85
           |||||||||||||||||||||||||
Sbjct  61  CTGCATTTCAATCCCGGGTAAGGTA  85
```

Figure 4

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