Original Article

Detection of *porphyromonas gingivalis* in oral potentially malignant disorders and oral squamous cell carcinoma using qRT-PCR: A comparative study

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Abstract

Background: Recent researches has shown a significant association between microorganisms and oral squamous cell carcinoma (OSCC). *Porphyromonas gingivalis*, the keystone pathogen in chronic periodontitis, is considered as an important potential etiologic agent of OSCC, but the underlying mechanisms by which *P. gingivalis* mediates OSCC progression remain poorly understood.

Aim: The aim of this study was to compare the levels of *P. gingivalis* in oral potentially malignant disorders, oral squamous cell carcinoma and normal oral mucosa using qRT-PCR.

Method and Material: Genomic DNA was extracted and quantified, and the expression of the *P. gingivalis* levels was done in 16 cases of oral potentially malignant disorders, 16 cases of oral squamous cell carcinoma and 16 cases of normal oral mucosa by quantitative real-time polymerase chain reaction (RT-qPCR).

Results: It was observed that there was an over expression of *P. gingivalis* in both oral potentially malignant disorders and oral squamous cell carcinoma with good mean cycle threshold (CT) value of 27.00 and 27.55, respectively. When comparing the levels of *P. gingivalis* in three groups, oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC) showed higher expression than normal mucosa and in between two groups OSCC showed higher expression than OPMD and the difference is statistically significant with *P* value less than 0.001.

Conclusion: Our findings suggest that there is an over expression of *P. gingivalis* in oral potentially malignant disorders and oral squamous cell carcinoma, compared to normal mucosa and highly expressed in OSCCs compared to OPMD. Increased levels of *P. gingivalis* in OPMDs and OSCCs may suggest the early event of tumorigenesis. Hence, it can be used as a valuable marker for early diagnosis, prognosis marker and in the identification of therapeutic targets.

Keywords: Oral microbiome, oral potentially malignant disorders, oral squamous cell carcinoma, *P. gingivalis*, polymerase chain reaction

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Submitted: 24-Apr-2024, **Revised:** 16-Aug-2024, **Accepted:** 08-Nov-2024, **Published:** 31-Dec-2024

Access this article online

Quick Response Code:

Website: https://journals.lww.com/JPAT/

DOI: 10.4103/jomfp.jomfp_119_24

How to cite this article: Suganya G, Sahana NS, Akalya P, Yadav ST, Suresh T, Chandrakala J. Detection of *porphyromonas gingivalis* in oral potentially malignant disorders and oral squamous cell carcinoma using qRT-PCR: A comparative study. J Oral Maxillofac Pathol 2024;28:583-8.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck, which accounts for approximately 180,000 deaths worldwide in 2018 (1.9% of total cancer cases).[1] In spite of therapeutic advances, the 5-year survival rate is approximately 50%.[2] OSCC is often preceded by oral potentially malignant disorders (OPMDs), but the mechanism by which OPMDs progress into cancer remains poorly known.

Relationship between human papillomavirus and oral carcinoma has also been established in many studies. Howeverr, the association between bacteria and human cancer is not common.[3] In recent studies, a considerable number of microorganisms have been proved to be strongly associated with human carcinogenesis, including *Helicobacter pylori* in gastric cancer,[4] *Fusobacterium nucleatum* in colorectal cancer,[5] and so on.

Porphyromonas gingivalis, a Gram-negative oral bacterium which acts as "keystone pathogen" in periodontitis, also involved in carcinogenesis in gastrointestinal tract cancers, such as colon cancer, pancreatic cancer, oesophageal cancer, and OSCC.[6] Chronic inflammation caused by bacteria is considered as one of the potential pathways for various stages of oral carcinogenesis and inflammation-induced DNA damage in epithelial cells caused by bacteria and endotoxins.[7,8] The relationship between *Porphyromonas gingivalis* and OSCC remains controversial. Hence, this study was undertaken to understand the role of *P. gingivalis* in OSCC. *P. gingivalis* levels may prove useful in early diagnosis and also acts as prognostic factor for oral potentially malignant disorders and therapeutic targets for OSCC.

MATERIAL AND METHODS

The study was conducted at the Department of Oral Pathology and Microbiology, Government Dental College and Research Institute, Bangalore, India.

The study protocol was approved by the Institutional Ethical Committee of Government Dental College and Research Institute, Bangalore, India, (Protocol No. GDCRI/IEC- ACM[2]/15/2022-2023).

Sample

A total number of 48 cases were selected from the archives of the Department of Oral Pathology, GDCRI Bangalore. Cases were divided into three groups,

Group I: 16 histopathologically diagnosed cases of oral potentially malignant disorders.

Group II: 16 histopathologically diagnosed cases of oral squamous cell carcinoma.

Group III: 16 histopathologically diagnosed normal oral mucosa.

METHOD

10 µm sections were cut from each formalin-fixed paraffin-embedded tissue block using a microtome and subsequently placed in 1.5 ml Eppendorf tubes [Figure 1a]. The sections were then deparaffinized by keeping the tissue section in the microwave oven at 65°C for 15 mins. Later 1 ml of xylene was added to the tissue sections in the Eppendorf tubes to dissolve the paraffin and centrifuged at 14000 rpm for 3 mins after which the supernatant was carefully pipetted out and the process was repeated thrice. Residual xylene was removed, and tissue rehydration was done by adding 1 ml of absolute ethanol centrifuged at 14000 rpm for 3 mins; later, the supernatant was removed by pipetting, and this process was also repeated thrice. The pellets were vacuum dried completely. The tissue pellets then were resuspended by adding 180 microlitre of lysis solution (AL) (DS0015) and incubated at room temperature for 30 minutes.

DNA extraction

DNA was extracted from deparaffinized tissue pellets by adding 20 micro litre of proteinase K solution (20 mg/ml) and mixed by vortexing and incubated at 56°C overnight [Figure 1b]. After overnight incubation, it was centrifuged at the speed of 20000 x g at 15000 rpm for 5–10 minutes and

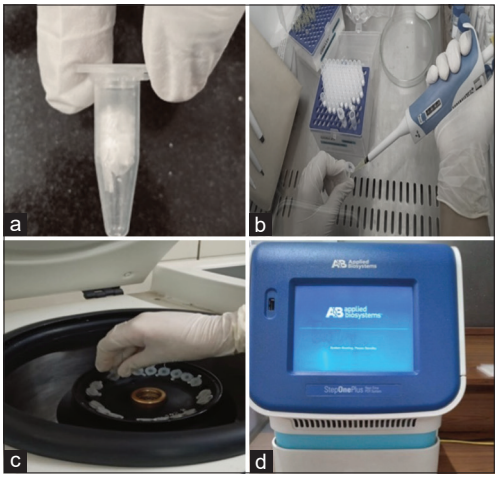


Figure 1: (a) A 10µm tissue section was placed in a 1.5ml Eppendorf tube (b)Proteinase K was added to the tube for enzymatic digestion facilitating the breakdown of proteins in the sample (c) The tube was then subjected to centrifugation to ensure thorough mixing and separation of the sample components (d) After preparation, the sample was processed using an RT-PCR machine for nucleic acid amplification and detection

the supernatant was transferred into a new 2.0 ml capped microcentrifuge tube [Figure 1c]. 20 micro litre of RNase A solution (DS0003) was added and mixed by pulse vortexing thoroughly for 15 secs and incubated for 2 mins at room temperature. 200 microlitre of lysis solution (C1) (DS0010) was added mixed by vortexing thoroughly for 15 seconds incubated at 70°C for 10 minutes. Later, 200 microlitre of absolute ethanol was added to lysate and mixed thoroughly by vortexing for 15 seconds. The lysate obtained was transferred to the HiElute Miniprep spin column and incubated at room temperature for 5 minutes. Later, the lysate was centrifuged at 6500 x g at 10000 rpm for 1 minute. After this, centrifugation flow was reloaded through liquid on to the column again and centrifuged at 10000 rpm for 1 minute. The flow through was discarded and placed into the same column. Wash solution is prepared and 500 microlitre of wash solution was added to the column and centrifuged at 10000 rpm for 1 minute [Figure 1c]. The flow through liquid was discarded, and the column was placed in same collection tube. Later, another 500 microlitre of diluted wash solution was added to the column and centrifuged at 20000 x g (15000 rpm) for 3 minutes. The collection tube containing flow through liquid was discarded, and the column was placed in a new 2.0 ml uncapped collection tube. DNA Elution was done by adding 50–100 microlitre of the elution buffer directly onto the column and incubated for 5 minutes at room temperature and centrifuged at 6500 x g (10000 rpm) for 1 minute.

DNA amplification

The specific primer sequences of both Forward (F) and Reverse (R) primer were commercially obtained. The total of 10 µl reaction volume per well was added in the 96 well-plate. 10 µl reaction volume constitutes 1 µl primer, 1 µl extracted DNA, 5 µl of master mix (SYBR Green) and 3 µl of nuclease free water. 96 well-plate with samples was sealed and loaded in an applied Biosystem Step One Plus TM Real-Time PCR system Thermal Cycling Block (S/N: 2720010242) [Figure 1d]. Initial denaturation at 98°C for 5 minutes, then 35 cycles of final denaturation at 94°C for 30 seconds, annealing at 58°C, extension at 72°C for 45 seconds and final extension at 72°C for 5 mins followed by cooling for 5 mins was set to run and final amplification graph was collected from PCR machine [Figure 2].

Statistical analysis

SPSS (Statistical Package for Social Sciences) version 21. (IBM SPASS statistics [IBM corporation: NY, USA]) was used to perform the statistical analysis.

Data were entered in the excel spread sheet. Descriptive statistics of the explanatory and outcome variables were calculated by mean, standard deviation for quantitative variables, frequency, and proportions for qualitative variables.

Inferential statistics like Kruskal-Wallis test (based on data distribution) was applied to compare the quantitative variables among the groups with *post hoc* Mann-Whitney test (based on data distribution) for intergroup comparison. The level of significance is set at 5%.

RESULTS

Data was subjected to Normalcy test (Shapiro Wilk test). Data showed skewed distribution. Hence non-parametric tests (Kruskal-wallis test with post-hoc Mann-whitney test) was applied. [Table 1].

The CT values of GAPDH and *P. gingivalis* were used to calculate the fold increase of *P. gingivalis* in OPMD, OSCC in relation to normal oral mucosal tissue. The mean fold increase of *P. gingivalis* in OPMD and OSCC was 18.88 and 27.78 [Table 2 and Graph 1].

Subsequently, the Kruskal-Wallis test (based on data distribution) was applied to compare the quantitative variables among the groups with the *post hoc* Mann–Whitney test for intergroup comparison. Value < 0.05 was considered statistically significant. The obtained *P* value was 0.001, which shows that the levels of *P. gingivalis* are increased in OPMD and OSCC compared to controls.

Pairwise comparison was done using the *post hoc* Mann–Whitney test and showed that there exists a statistically highly significant difference in the mean fold increase of *P. gingivalis* expression between OPMD group and control group ($P < 0.001$) [Table 3 and Graph 2].

DISCUSSION

In recent years, association of oral microbiome and development of cancer has been a focus of research. *P. gingivalis* is regarded as a keystone pathogen of periodontitis, it has the ability to disrupt the host immune response, and it has been reported to be associated with OSCC progression. The virulence of *P. gingivalis* has been linked to various potential factors associated with its cell surface, including fimbriae, capsules, proteases, haemagglutinins lipopolysaccharides, and major outer membrane proteins. *P. gingivalis* also has the capacity to invade and penetrate various epithelial cells.

Few experimental and clinical studies have shown various degrees of associations between *P. gingivalis* and oral cancer and gastrointestinal tract cancers, including

oesophageal cancer and pancreatic cancer.[1] Therefore, we investigated whether it does play an important role in OSCC progression. Tissue specimens are advantageous compared to saliva when studying the microbial shifts as it provides subsite specific composition. PCR detection is highly sensitive and specific technique especially for bacterial identification because it is not influenced by microorganism growth like culture.[2]

Recent researches mentioned several hypotheses for the molecular mechanism of *P. gingivalis* in OSCC progression. *P. gingivalis* resides in the subgingival site and is a Gram-negative anaerobic bacterium taking part in promoting antiapoptotic pathway by increasing the expression of antiapoptotic genes and by blocking the proapoptotic genes [Figure 3].

P gingivalis main virulence factors which permits oral epithelial cell invasion are capsule polysaccharides which increases biofilm production and deregulate cell cycle and signalling pathways, cysteine proteases (gingipains R and K) that destroys extra cellular matrix, and fimbria that joins to cellular superficial β1 integrins.

Fimbriae are also considered to be critical virulence factors of *P. gingivalis*. FimA fimbriae (long fimbriae) are primarily composed of polymers of the FimA protein encoded by the fimA gene, whereas Mfa1 fimbriae (short fimbriae) are mostly composed of the Mfa1 protein encoded by the mfa1 gene. Long and short fimbriae induce various cytokine expressions, such as IL-1, IL-6, and TNF-α, which result in alveolar bone resorption.

In this way, *P gingivalis* deceives and evades the immune system, creating a chronic inflammatory stimulus that promotes cell multiplication and bacterial invasion. Furthermore, inflammation and specifically chronic inflammation in periodontal disease might be related to OSCC, as well as to other malignancies.[10,11]

There are different steps in which *P gingivalis* may be involved, such as epithelial–mesenchymal transition of malignant epithelial cells, neoplastic cell growth, along with their proliferative and invasive capacities

The bacterium upregulates specific receptors on OSCC cells and keratinocytes, induces epithelial-to-mesenchymal (EMT) transition of normal oral epithelial cells activates metalloproteinase-9 and interleukin-8 in cultures of the carcinoma cells. The underlying molecular mechanisms have not yet been fully understood, but recent studies shown that long-term chronic bacterial infection promotes OSCC through direct interaction with cancerous and precancerous oral epithelial cells via toll-like receptors.

Members of the oral microbiota may directly stimulate OSCC proliferation and induce expression of key

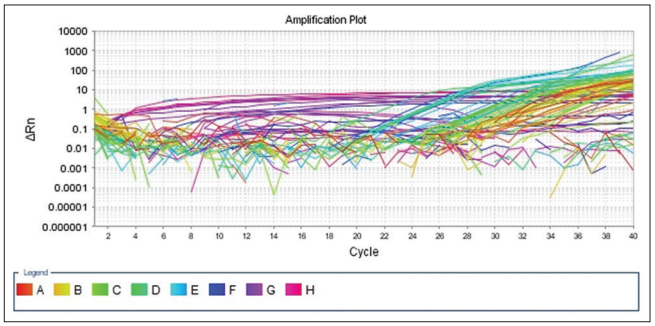
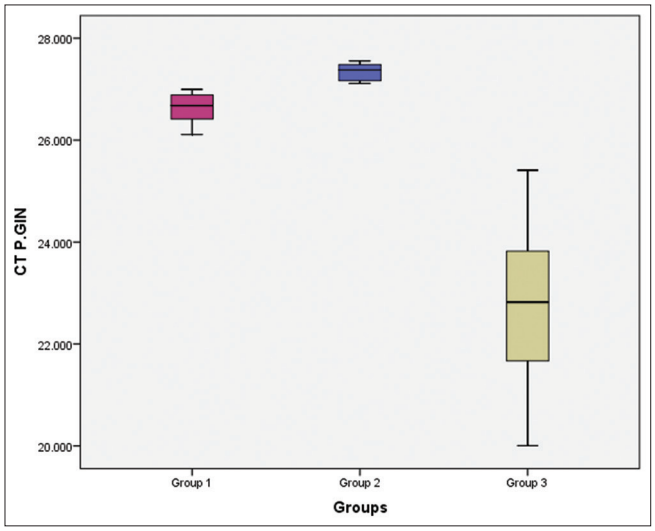
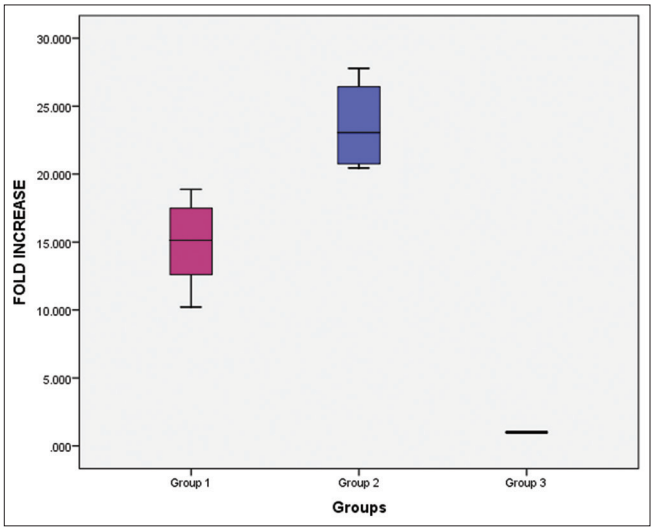


Figure 2: Amplification plot



Graph 1: Mean CT values between the three groups



Graph 2: Fold increase between the groups

Table 1: Tests of normality

Groups		Tests of Normality[c]					
		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	*P*	Statistic	Df	*P*
Group 1	CT P.GIN	0.174	16	0.200*	0.924	16	0.194
	Delta CT	0.114	16	0.200*	0.963	16	0.713
	double delta CT	0.174	16	0.200*	0.924	16	0.194
	Fold increase	0.176	16	0.200*	0.936	16	0.298
Group 2	CT P.GIN	0.183	16	0.155	0.872	16	0.029
	Delta CT	0.165	16	0.200*	0.919	16	0.160
	double delta CT	0.183	16	0.155	0.872	16	0.029
	Fold increase	0.215	16	0.046	0.847	16	0.012
Group 3	CT P.GIN	0.092	16	0.200*	0.985	16	0.991
	Delta CT	0.124	16	0.200*	0.947	16	0.444
	double delta CT	0.292	16	0.001	0.702	16	0.000

*. This is a lower bound of the true significance. a. Lilliefors Significance Correction. c. FOLD INCREASE is constant in one or more split files. It has been omitted

Table 2: Comparison of the groups using kruskal-wallis

Dependent variable	Group	Minimum	Maximum	Median	IQR	*P*
CT P. Ging	Group 1	26.11	27.00	26.68	0.49	0.001*
	Group 2	27.11	27.55	27.38	0.34	
	Group 3	20.01	25.41	22.82	2.24	
Delta CT	Group 1	0.80	9.77	4.12	3.13	0.001*
	Group 2	0.15	7.77	2.51	3.03	
	Group 3	1.00	11.98	8.11	5.72	
Double Delta CT	Group 1	-4.24	-3.35	-3.92	0.49	0.001*
	Group 2	-4.80	-4.35	-4.62	0.34	
	Group 3	-2.65	11.45	0.27	2.39	
Fold Increase	Group 1	10.22	18.88	15.13	5.01	0.001*
	Group 2	20.45	27.78	23.05	6.07	
	Group 3	-	-	-	-	

*significant

TABLE 3: Inter-group comparison using post-hoc mann-whitney test

Group	Mann whitney (U & P value)	CT P.GIN	Delta CT	double delta ct	Fold increase
Group 1 Vs	*U*	0.000	77.000	0.000	0.000
Group 2	*P*	0.001*	0.055	0.001*	0.001*
Group 1 Vs	*U*	0.000	55.000	0.001*	0.001*
Group 3	*P*	0.001*	0.006	0.000	0.000
Group 2 Vs	*U*	0.000	30.000	0.001*	0.001*
Group 3	*P*	0.001*	0.001*	0.001*	0.001*

* *P* value set significant at 0.05/3=0.0125

molecules (e.g. NFkB, IL-6-STAT3, cyclin D1, MMP-9, and the bacterial gingipains) that are implicated in tumorigenesis.

In 2011, Katz *et al*.[12] found that *P. gingivalis* expression was much higher in OSCC tissue than normal tissue by comparing samples from 10 cases of OSCC and 5 healthy subjects using IHC analysis; they also examined the symbiotic bacterium *Streptococcus gordonii* as a reference and found no difference. Although the sample size of that study was small, this was the first report of a significant positive correlation between *P. gingivalis* detection and the occurrence of OSCC.

In addition, Chang *et al*. found that the detection rate of *P. gingivalis* in gingival squamous cell carcinoma was about 45%, that of tongue squamous cell carcinoma was about 40%, and that of normal gingivalis tissue was about 20% by analysing the samples from patients with OSCC in China; the difference between OSCC and normal tissue was statistically significant.[13]

In 2020, Nehad M. Monier *et al*.[2] done a study on detection of the periodontal pathogen *P. gingivalis* in oral squamous cell carcinoma by conventional PCR in 25 oral cancer tissue specimens versus 25 healthy oral mucosal tissue and concluded that *P. gingivalis* was significantly higher in control group. Bacterial presence was not found to be correlated with tumour progression.

In 2021, Jinyu Kong *et al*.[6] done a study on frequencies of *P. gingivalis* Detection in Oral-Digestive Tract Tumors by IHC, PCR and analysed the correlation between *P. gingivalis* detection and clinicopathological characteristics, prognosis of oral and oesophageal carcinoma. The IHC results showed that the positive rates of *P. gingivalis* were 60.00, 46.00, 20.00, 6.67 and 2.86% in oral, oesophagus, cardiac, stomach and colorectal cancer tissues, respectively. Likewise, PCR results showed the rates of 56.00, 42.00, 16.67, 3.33, and 2.86%, respectively.

Through our study, we are reporting that *P. gingivalis* was expressed more in OPMDs and OSCCs with a mean fold increase of 18.88 ± 5.01 and 27.78 ± 6.07 and comparative to normal oral mucosa [Graph 2]. Our study showed that the expression was more in OSCCs compared to OPMDs and normal oral mucosa indicating the strong association with OSCC. *P. gingivalis* levels were more in OPMD compared to normal oral mucosa suggesting that it is an early change in OSCC and plays a role in tumorigenesis. Hence, *P. gingivalis* expression will be useful in early diagnosis of oral potentially malignant disorders. Considering all these data along with the present study results, it can be stated that the evaluation of *P. gingivalis* expression can act as a diagnostic and prognostic marker as well as for targeted therapies along with conventional treatment.

CONCLUSIONS

This study showed that *P. gingivalis* levels are more in OSCC and OPMD, and between the two groups, it was expressed more in OSCC. *P. gingivalis* in conjunction with established risk factors e.g. alcohol consumption may play a role in increased cancer development. It

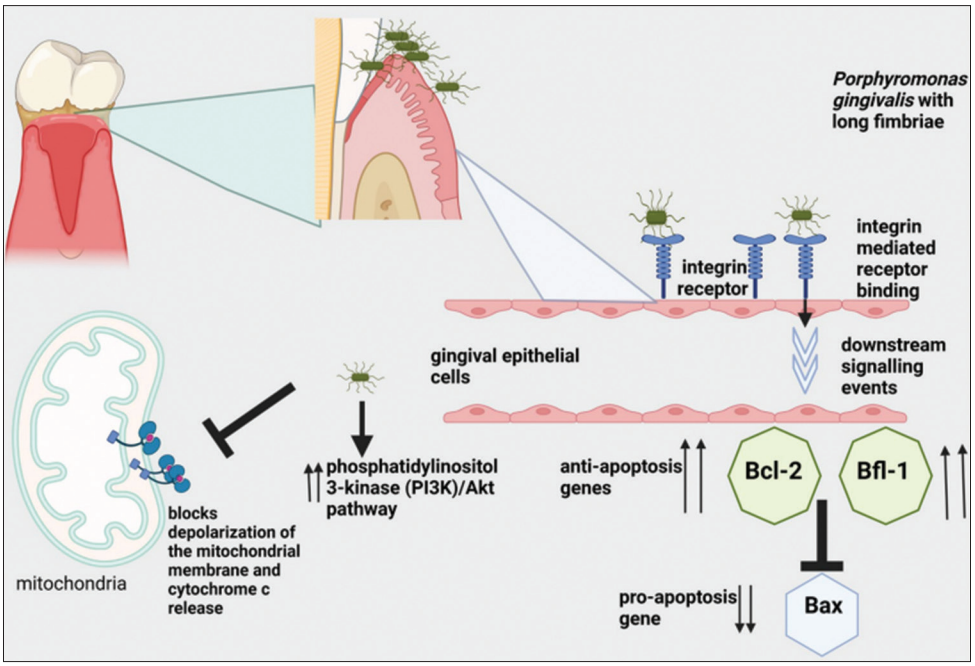


Figure 3: *P. gingivalis* activates PI3/Akt pathway, which enhances cancer [IMAGE COURTESY:[9]]

has been suggested that in the presence of risk factors such as alcohol and tobacco abuse; the commensal oral microbiota may act synergistically in the oral cancer pathogenesis. *P. gingivalis* could play an important role in promoting the occurrence and development of OSCC, and it is considered as an important risk factor. *P. gingivalis* may be used as a biomarker for early diagnosis and also to evaluate the malignant degree and prognosis of OSCC.

Recently, scientists showed that microorganisms can be used for cancer treatment, but whether *P. gingivalis* is beneficial to human health remains unclear. The pathogenic mechanism of *P. gingivalis* must be further explored to provide a new direction for cancer prevention, treatment, and to improve the health level and quality of life of patients suffering from this disease.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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