Bioinformatics and immunohistochemistry analyses of expression levels and clinical significance of CXCL2 and TANs in an oral squamous cell carcinoma tumor microenvironment of *Prophyromonas gingivalis* infection

ZHI-CHEN GUO[1], SAKENDEKE JUMATAI[1], SI-LI JING[2], LU-LU HU[1], XIN-YU JIA[1] and ZHONG-CHENG GONG[1,3]

[1]Oncological Department of Oral and Maxillofacial Surgery, The Affiliated Stomatology Hospital of The First Affiliated Hospital of Xinjiang Medical University;
[2]Department of Ophthalmology, The First Affiliated Hospital of Xinjiang Medical University;
[3]Xinjiang Uygur Autonomous Region Institute of Stomatology, Urumqi, Xinjiang Uyghur Autonomous Region 830054, P.R. China

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Abstract. The present study aimed to detect the immunoexpression and clinical significance of *Porphyromonas gingivalis* (*P. gingivalis*) in the tumor microenvironment (TME) of oral squamous cell carcinoma (OSCC). The immunoexpression of *P. gingivalis* in OSCC tissues was detected via immunohistochemistry (IHC) after *P. gingivalis* was infected into the TME of OSCC. To identify the differentially expressed genes in the carcinogenesis and progression of OSCC with *P. gingivalis* infection, microarray datasets (GSE87539 and GSE138206) were downloaded from the Gene Expression Omnibus database. The immunoexpression levels of C-X-C motif chemokine ligand 2 (CXCL2) and tumor-associated neutrophils (TANs) were also evaluated via IHC, and the immunoexpression levels of all three clinical variables were analyzed using χ^2 or Fisher's exact tests. The survival rates were calculated using the Kaplan-Meier method and the survival curves were compared using log-rank tests. Predominantly strong immunoexpression of *P. gingivalis* was identified in OSCC samples. CXCL2 was considered to be a differential gene in the two datasets. Immunoexpression of *P. gingivalis* was positively associated with CXCL2 and TANs expression. Furthermore, *P. gingivalis* was associated with survival status (P<0.001) and differentiation (P<0.001). CXCL2 was associated with age (P=0.038) and survival status (P=0.003), while TANs were associated with T stage (P=0.015) and clinical stage (P=0.002). These clinical variables were considered to be independent risk factors for the poor prognosis of patients with OSCC. Collectively, the results suggested that the immunoexpression of *P. gingivalis* may be positively associated with CXCL2 and TANs. In addition, the strong immunoexpression levels of *P. gingivalis*, CXCL2 and TANs may be associated with a poor prognosis in patients with OSCC.

Correspondence to: Professor Zhong-Cheng Gong, Oncological Department of Oral and Maxillofacial Surgery, The Affiliated Stomatology Hospital of The First Affiliated Hospital of Xinjiang Medical University, 137 Li Yu Shan South Road, Urumqi, Xinjiang Uyghur Autonomous Region 830054, P.R. China
E-mail: gump0904@aliyun.com

Abbreviations: P. gingivalis, *Porphyromonas gingivalis*; CXCL2, C-X-C motif chemokine ligand 2; TME, tumor microenvironment; IHC, immunohistochemistry; OSCC, oral squamous cell carcinoma; DEGs, differentially expressed genes; TANs, tumor-associated neutrophils; GEO, Gene Expression Omnibus; CSR, cumulative survival rate

Key words: P. gingivalis, OSCC, TANs, CXCL2, prognosis

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the head and neck (1), and is associated with high incidence, invasion and metastasis rates, as well as a poor prognosis. In the United States, ~50,000 individuals suffered from OSCC and 8,000 individuals died in 2013 (2). OSCC has gradually become a serious problem worldwide, despite the increase in basic research and the rapid development of clinical treatment in the past few decades. Furthermore, the 5-year survival rate of this disease has not significantly improved (3). With the identification of a causal link between *Helicobacter pylori* (*H. pylori*) infection and the occurrence of gastric tumors in the 1900s (4), an increased understanding has been achieved regarding the association between bacteria and tumors. Nevertheless, how oral microorganisms influence the tumor microenvironment (TME), and how they promote tumor development during tumor progression, remains unknown (1). It has been reported that a large number of pro-inflammatory cytokines secreted by microorganisms can transform tumor cells into more aggressive phenotypes by regulating oncogenes (5). Tumor growth and metastasis are not only affected by neoplastic cells, but also by the TME (6). Surgery combined

with chemotherapy is an effective treatment for OSCC (1). However, chemotherapy is not a first-line therapy for OSCC, since most OSCC cases develop resistance to chemotherapeutic reagents (7). Periodontitis has been suggested to be associated with the TME of OSCC, which could be involved in the development of chemotherapy resistance in OSCC (7). Therefore, further investigations examining the regulatory mechanism of the TME and identifying novel biomarkers to optimize patient selection for this therapy have gained clinical significance and theoretical value.

Porphyromonas gingivalis (*P. gingivalis*) is a key pathogen in periodontal disease, and is known as an independent microorganism risk factor for increased tumor mortality (8). *P. gingivalis* is a special oral pathogen and a potentially independent microbiological factor that increases the risk of mortality in patients with OSCC (9). A previous study revealed the presence of the *P. gingivalis* antigen in the tissues of gingival squamous cell carcinoma (10). An abnormal increase in the number of *P. gingivalis* is an important factor in causing the imbalance of the local microecology of the oral cavity (7). The dominant bacterial pathogen in periodontitis is *P. gingivalis*, which affects the condition of the TME, increasing the likelihood of OSCC development (11).

Chemokines are a small molecular protein family that serve a role in chemical signaling during cell activation and differentiation, as well as in the process of movement. Furthermore, chemokines and their signaling receptors are important in the TME of OSCC (12). According to a previous study, *P. gingivalis* induces immune cells to secrete chemokines [including C-X-C motif chemokine ligand (CXCL)1, CXCL2, CXCL5 and CXCL8] and promotes tumor growth (13). CXCL2 is a type of small molecular pro-inflammatory chemokine and a member of four subfamilies (C, CC, CXC and CX3C), serving a role in coding protein secretion, immune regulation and the promotion of tumor angiogenesis (14). CXCL2 is highly expressed in breast, colon, prostate and liver tumors, and is closely associated with tumor growth, invasion and distant metastasis (15). However, the other biological functions of CXCL2 in the *P. gingivalis*-infected TME of OSCC remain unknown.

Polymorphonuclear neutrophils (PMNs) are the primary defense line of the immune system, and are the most abundant white blood cells in the peripheral blood circulation (16). Tumor-associated neutrophils (TANs) are infiltrates in the TME, and there are two polarized manifestations of TANs: Defined as the N1 phenotype, one has the ability of typical tumor inhibition, while another, defined as the N2 phenotype, has typical tumor promotion, the differentiation of which depends on the factors of the TME (17,18). In addition, $CD66b^+$ has been assessed as a PMN marker in gastric tumors using immunohistochemistry (IHC), and $CD66b^+$ has been revealed to be a reliable marker to identify the phenotype of TANs that promote tumor growth (19). It has also been revealed that TANs are primarily activated by CXC chemokines (20).

In order to analyze the immunoexpression and clinical significance of CXCL2 and TANs in the TME of *P. gingivalis* infection, the immunohistochemistry evaluation, bioinformatics analyses and statistical analysis were performed.

Materials and methods

Patient selection. The present study included 205 surgical specimens from patients [age range, 36-90 years; mean age, 63 years; 103 men (50.2%); 102 women (49.8%); 76 patients <60 years (37.1%); 129 patients ≥60 years (62.9%)] who underwent surgical treatment for primary OSCC at the Oncology Department of Oral and Maxillofacial Surgery of The First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) between March 2007 and March 2019. Surgical resection was performed in all 205 patients with OSCC. The TNM and clinicopathological classification and staging of patients with OSCC were performed according to the American Joint Committee on Cancer (AJCC) guidelines (21). The inclusion criteria were as follows: i) patients with OSCC located in the tongue, buccal cavity, lip, floor of the mouth, or gingival and retromolar area as confirmed via biopsy; ii) patients who did not undergo any treatment before; and iii) patients with completed clinical data and follow-up. Clinical data, including age, sex, survival status, differentiation, tobacco and alcohol consumption, TNM stage, clinical stage, recurrence, periodontal condition, treatment (surgery, radiotherapy and/or chemotherapy, post-operative adjuvant radiotherapy or chemotherapy) and tumor size, were collected. In addition, the present study included 20 cases of benign tumor and non-tumor patients [age range, 23-48 years; mean age, 33 years; 10 men (50.0%); 10 women (50.0%)] who underwent surgical treatment at the Oncology Department of Oral and Maxillofacial Surgery of The First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) between May 2017 and May 2019. The inclusion criteria were as follows: i) Patients with benign tumors located in the oral cavity; ii) patients with benign tumors located in the parotid gland, sublingual gland and submandibular gland; iii) patients with inflammatory lesions requiring surgical removal. The present study was approved by the ethics review board of the First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) at the original time of data collection (approval no. IACUC20180411-13) and written consent was obtained at the original time of data collection.

Bioinformatics analyses. Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) is a public functional genomics data repository of high-accuracy gene expression data, chips and microarrays. A total of two gene expression datasets, GSE87539 (22) and GSE138206 (23), were downloaded from GEO (Affymetrix GPL570 platform; Affymetrix Human Genome U133 Plus 2.0 Array; Thermo Fisher Scientific, Inc.). The probes were converted into the corresponding gene symbols according to the annotation information in the platform. The GSE87539 dataset contained 3 samples of non-infected oral epithelial cells and 3 samples of *P. gingivalis*-infected oral epithelial cells. The GSE138206 dataset contained 6 OSCC samples and 6 non-cancerous samples.

The differentially expressed genes (DEGs) between GSE87539 and GSE138206 samples were screened using GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r). GEO2R is an interactive web tool that allows users to compare ≥2 datasets in a GEO series to identify DEGs across the experimental

conditions. Hierarchical clustering of hub genes was performed using UCSU Cancer Genomics Browser (http://genome-cancer.ucsc.edu) (24). The adjusted P-values (adj. P) and Benjamini and Hochberg false discovery rate were applied to provide a balance between the discovery of statistically significant genes and limitations of false-positives. The probe sets without corresponding gene symbols or genes with >1 probe set were removed or averaged, respectively. Log fold-change >1 and adj. P<0.01 were considered to be statistically significant.

IHC evaluation. A total of 205 OSCC samples from patients were immersed in 10% neutral buffered formalin for fixation for 3 days at room temperature. A total of 205 paraffin-embedded OSCC samples from patients and consecutive 4-μm-thick sections cut from paraffin blocks were used for IHC evaluation. The expression levels of *P. gingivalis* were evaluated, and 119 samples exhibited strong expression. These 119 samples were used in the evaluation of the expression levels of CXCL2 and TANs. Non-cancerous samples were obtained from patients who suffered from benign tumors or inflammatory disease at the Oncological Department of Oral and Maxillofacial Surgery, The Affiliated Stomatology Hospital of The First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) and maintained at the Institute of Stomatology of Science and Technology Building of the First Affiliated Hospital of Xinjiang Medical University (Urumqi, China). The IHC procedure was as follows: Slides were heated at 65˚C for 2 h. Sections that adhered to the slides were deparaffinized in xylene and rehydrated in a gradient ethanol (50% ethanol for 10 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 95% ethanol for 10 min, 100% ethanol for 10 min three times), followed by submerging into EDTA antigenic retrieval buffer, treatment with 3% hydrogen peroxide for 15 min at room temperature and incubation with 1% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C. The samples were then incubated with anti-*P. gingivalis* (dilution, 1:200; Dia-An, lnc; this antibody is not a commercial product, and it was prepared at the time), anti-CXCL2 (dilution, 1:500; cat. no. bs-1162R; BIOSS) and anti-TANs ($CD66b^+$; dilution, 1:500; cat. no. ab197678; Abcam) antibodies overnight at 4˚C. After being washed with PBS with 1% Tween, the slides were incubated with secondary antibodies [Goat Anti-Mouse IgG H & L (HRP); dilution, 1:2,000; cat. no. ab205719; Abcam; and Goat Anti-Rabbit IgG H & L (HRP); dilution, 1:2,000; cat. no. ab205718; Abcam] for 1 h at 37˚C, followed by incubation with streptavidin horseradish peroxidase complex (Thermo Fisher Scientific, Inc.). The sample sections were then immersed in 3,3'-diaminobenzidine for 5 min at room temperature, counterstained with 10% Mayer's hematoxylin for 1 min at room temperature, dehydrated and mounted.

Each tumor specimen was observed by light microscopy and examined under 10 microscopic fields with a digital camera (AxiocamMRc; Zeiss AG) attached to a microscope (magnification, x200; Axioskop 2 Plus Zeiss AG) to evaluate the immunoexpression levels of *P. gingivalis,* CXCL2 and TANs. Two experienced pathologists evaluated the three immunoexpression profiles of cancerous tissues and non-cancerous tissues. The final score was determined by multiplying the immunostaining intensity by the percentage of positive immunostaining cells. Subsequently, the cancerous samples were classified into three groups: 0, absent immunostaining; 1, weak immunostaining; and 2, strong immunostaining. The proportion of positive cells was classified as follows: 0, 0; 1, 1-25; 2, 26-75; 3, 76-100%. The final staining score was calculated by multiplying the staining intensity score by extent of staining score. A final staining score of ≥3 was considered positive, and others were classified as low expression.

Statistical analysis. Statistical analysis was performed using SPSS statistical software (version 21.0; IBM Corp.). Data are presented as value (%) and the association of *P. gingivalis*, CXCL2 and TANs immunoexpression levels in neoplastic cells with the clinicopathological variables was determined using χ^2 or Fisher's exact tests (two-sided). For these analyses, the absence and weak immunoexpression levels were grouped together, obtaining final groups of absent/weak tumor immunoexpression and strong neoplastic immunoexpression. The cumulative survival rate (CSR) probability in 10 years was estimated using the Kaplan-Meier method, and survival curves were compared via a log-rank test. The follow-up period considered for cumulative survival consisted of the time between the date of surgery and mortality or the date of the last information collection regarding the patient. Cox regression analysis of the survival data was performed to test the statistical significance of regression coefficients. P<0.05 was considered to indicate a statistically significant difference.

Results

Study population. Between March 2007 and March 2019, a total of 205 samples from patients who were newly diagnosed with OSCC were retrospectively analyzed. There were slightly more men than women (50.2%) and patients with an age of ≥60 years (62.9%). Alcohol (18.0%) and tobacco (28.3%) consumption were considered as partial risk factors in patients.

With regard to survival status, the majority of patients in the present study were alive at the time of retrospective analysis (71.7%). Furthermore, the patients were clinically classified as stage I-II (54.1%) and III-IV (45.9%), and the TNM stages were classified as T1-T2 (65.9%) and T3-T4 (34.1%), and N0 (70.7%) and N(+) (29.3%). There were no patients with distant metastases at the time of physical examination (data not shown).

Among the enrolled patients, 67.3 and 23.9% were classified as well and moderately differentiated, while 8.8% were classified as poorly differentiated, according to the histopathological grade of tumor malignancy as described by Bryne *et al* (25). Recurrence occurred in 14.6% of patients, and there were 101 patients (49.3%) with poor periodontal condition. Only 13 patients (6.3%) underwent no therapy, 90 patients (43.9%) underwent surgical therapy, 14 patients (6.8%) received radiotherapy and/or chemotherapy and 88 patients (42.9%) received comprehension therapy (post-operative adjuvant radiotherapy and/or chemotherapy; Table I).

IHC expression of P. gingivalis in TME of OSCC. IHC expression of *P. gingivalis* in OSCC was weakly positive in 86 samples, while the expression was strongly positive in 119 samples. *P. gingivalis* immunoexpression was predominant in the cytoplasm of neoplastic cells. The strong immunoexpression

Table I. General information of 205 patients with oral squamous cell carcinoma.

Variable	No. of patients (%)
Sex	
Male	103 (50.2)
Female	102 (49.8)
Age, years	
<60	76 (37.1)
≥60	129 (62.9)
Survival status	
Alive	147 (71.7)
Dead	58 (28.3)
Tobacco consumption	
Yes	58 (28.3)
No	147 (71.7)
Alcohol consumption	
Yes	37 (18.0)
No	168 (82.0)
Clinical stage	
I-II	111 (54.1)
III-IV	94 (45.9)
T stage[a]	
T1-2	135 (65.9)
T3-4	70 (34.1)
N stage[a]	
N0	145 (70.7)
N(+)	60 (29.3)
Differentiation	
Well	138 (67.3)
Moderate	49 (23.9)
Poor	18 (8.8)
Recurrence	
Yes	30 (14.6)
No	175 (85.4)
Periodontal condition	
Well	104 (50.7)
Poor	101 (49.3)
Treatment	
None	13 (6.3)
Surgery	90 (43.9)
Chemotherapy + radiotherapy	14 (6.8)
Comprehensive	88 (42.9)

[a]According to the 7th American Joint Committee on Cancer/Union for International Cancer Control staging system.

of *P. gingivalis* was detected in carcinoma nests, while negative immunoexpression was observed in non-cancerous samples (Fig. 1).

Clinical variables, including the survival status, differentiation, tobacco consumption, T stage, N stage, clinical stage, periodontal condition, tumor size and treatment methods.

Table II. Immunohistochemical expression of *P. gingivalis* in samples from 205 patients with oral squamous cell carcinoma according to clinical data and follow-up.

	P. gingivalis		
Variable	Weak, n (%)	Strong, n (%)	P-value
Sex			0.233
Male	39 (45.3)	64 (53.8)	
Female	47 (54.7)	55 (46.2)	
Age, years			0.361
<60	35 (40.7)	41 (34.5)	
≥60	51 (59.3)	78 (65.5)	
Survival status			<0.001[a]
Alive	75 (87.2)	72 (60.5)	
Dead	11 (12.8)	47 (39.5)	
Differentiation			<0.001[a]
Well	70 (81.4)	68 (57.1)	
Moderate	16 (18.6)	33 (27.7)	
Poor	0 (0.0)	18 (15.1)	
Tobacco consumption			0.047[a]
Yes	18 (20.9)	40 (33.6)	
No	68 (79.1)	79 (66.4)	
Alcohol consumption			0.346
Yes	12 (14.0)	24 (20.2)	
No	74 (86.0)	95(79.8)	
T stage[b]			<0.001[a]
T1-2	73 (84.9)	62 (52.1)	
T3-4	13 (15.1)	57 (47.9)	
N stage[b]			0.011[a]
N0	69 (80.2)	76 (63.9)	
N(+)	17 (19.8)	43 (36.1)	
Clinical stage			<0.001[a]
I-II	63 (73.3)	48 (40.3)	
III-IV	23 (26.7)	71 (59.7)	
Recurrence			0.301
Yes	10 (11.6)	20 (16.8)	
No	76 (88.4)	99 (83.2)	
Periodontal condition			0.018[a]
Well	52 (60.5)	52 (43.7)	
Poor	34 (39.5)	67 (56.3)	
Tumor size, cm			0.007[a]
<2.9	51 (59.3)	48 (40.3)	
≥2.9	35 (40.7)	71 (59.7)	
Treatment			0.007[a]
None	2 (2.3)	11 (9.2)	
Surgery	49 (57.0)	41 (34.5)	
Chemotherapy + radiotherapy	4 (4.7)	10 (8.4)	
Comprehensive	31 (36.0)	57 (47.9)	

[a]P<0.05. [b]According to the 7th American Joint Committee on Cancer/Union for International Cancer Control staging system. P-values were determined using χ^2 or Fisher's exact tests. *P. gingivalis*, *Porphyromonas gingivalis*.

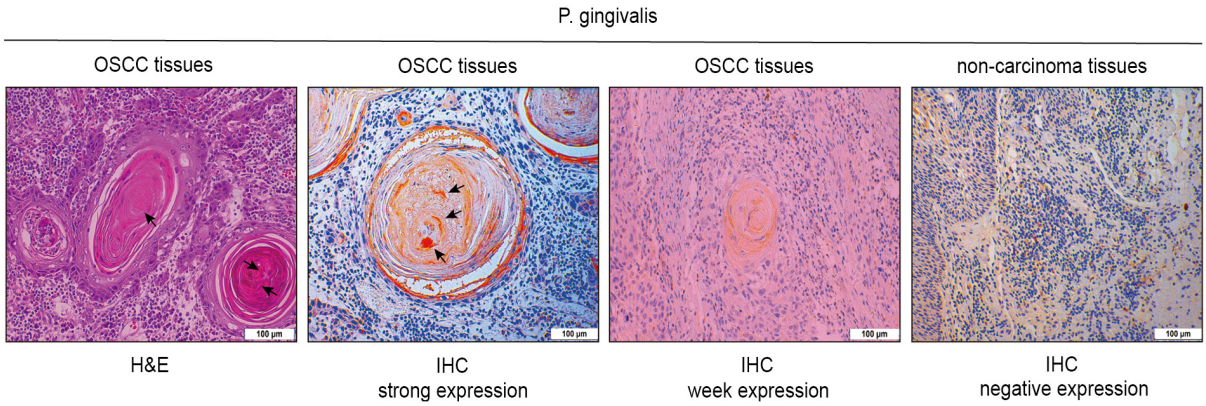

Figure 1. H&E staining of *P. gingivalis* in OSCC tissues, strong *P. gingivalis* expression in OSCC tissues, weak *P. gingivalis* expression in OSCC tissues and negative *P. gingivalis* expression in non-carcinoma tissues. Scale bars, 100 μm. *P. gingivalis*, *Porphyromonas gingivalis*; OSCC, oral squamous cell carcinoma; IHC, immunohistochemistry.

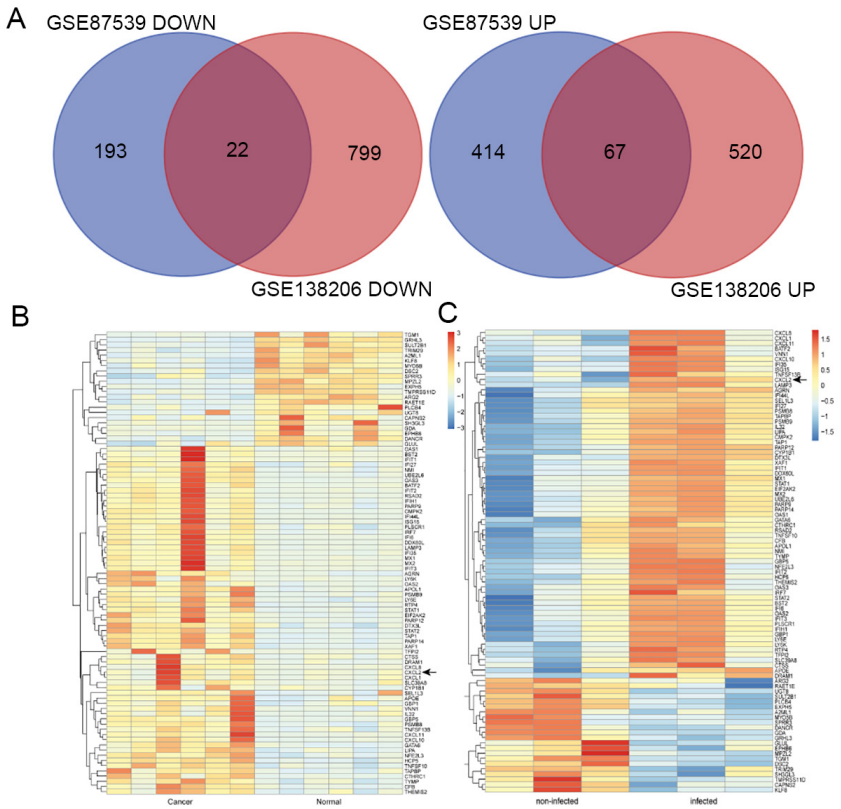

Figure 2. Venn diagrams and heat map of DEGs. CXCL2 was one of the DEGs included in the two datasets. (A) DEGs with a fold-change >2 and P<0.01 were selected in the gene expression profile datasets (GSE87539 and GSE138206). The two datasets had an overlap of 89 genes, including 67 upregulated genes and 22 downregulated genes. (B) The six samples on the left were carcinoma tissues and the six samples on the right were non-carcinoma tissues. Upregulation of genes is indicated in red, and downregulation of genes is indicated in blue. (C) Hierarchical clustering of DEGs was performed using UCSC. The three samples on the left were non-infected oral epithelium tissues and the three samples on the right were *Porphyromonas gingivalis*-infected tissues (black arrows indicate CXCL2). DEGs, differentially expressed genes; CXCL2, C-X-C motif chemokine ligand 2.

The result revealed that death, poor differentiation, tobacco consumption, advanced T stage, N stage and clinical stage, poor periodontal condition, large size of tumor and no treatment exhibited significant associations with strong immunoexpression of *P. gingivalis* (Table II).

Identification of CXCL2 as a DEG in the TME of OSCC infected with P. gingivalis by bioinformatics analyses. After standardizing the microarray results, the DEGs (26,469 in GSE87539 and 9,443 in GSE138206) were identified (data not shown). The overlap between the two datasets containing 89 genes is presented in the Venn diagram (Fig. 2A), and included 67 upregulated genes and 22 downregulated genes.

Hierarchical clustering demonstrated that CXCL2 was a DEG between GSE87539 (*P. gingivalis*-infected oral epithelial samples compared with non-infected oral epithelial samples) and GSE138206 (cancerous samples compared with non-cancerous samples; Fig. 2B and C).

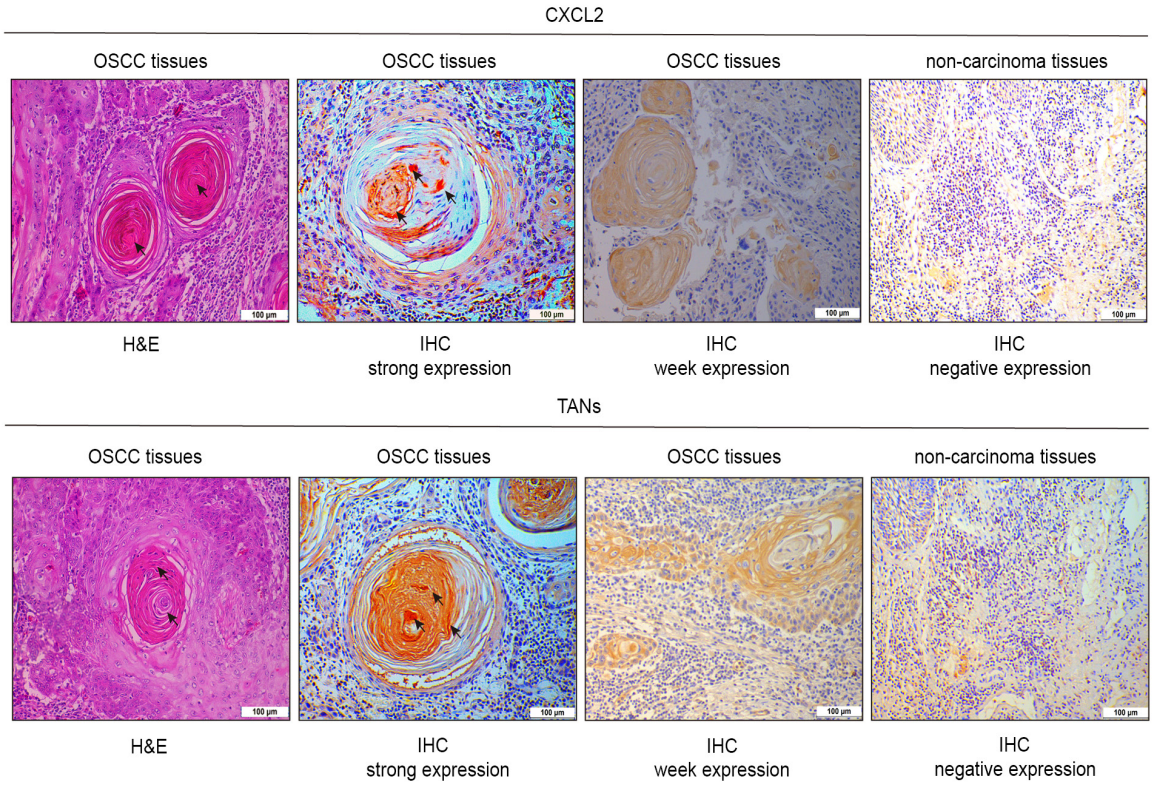

Figure 3. H&E staining of CXCL2 and TANs in OSCC tissues, strong CXCL2 and TANs expression in OSCC tissues, weak CXCL2 and TANs expression in OSCC tissues, and negative CXCL2 and TANs expression in non-carcinoma tissues. Scale bars, 100 μm. OSCC, oral squamous cell carcinoma; CXCL2, C-X-C motif chemokine ligand 2; TANs, tumor-associated neutrophils; IHC, immunohistochemistry.

IHC expression of CXCL2 and TANs in P. gingivalis-infected TME of OSCC. A total of 119 samples with high expression levels of *P. gingivalis* were selected for IHC examination of CXCL2 and TANs. In 119 samples with high *P. gingivalis* expression, CXCL2 and TANs were weakly positively expressed in 30 and 38 samples, respectively, but were strongly positively expressed in 89 and 81 samples, respectively. CXCL2 was strongly positively expressed in the OSCC samples, but was negatively expressed in the non-cancerous samples (Fig. 3).

$CD66b^+$ TANs are associated with a poor prognosis of patients with cervical cancer (26). In the present study, $CD66b^+$ TANs exhibited strong positive expression in OSCC samples and negative expression in non-cancerous samples (Fig. 3).

Clinical variables, including age, survival status, T stage, tumor size and treatment. The result revealed that high age, death, advanced T stage, large size of tumor and no treatment exhibited significant associations with strong CXCL2 expression.

In the present study, clinical variables, including advanced T stage and clinical stage, were statistically associated with strong expression of TANs (Table III). The present results indicated that the expression levels of *P. gingivalis* were positively associated with the expression levels of CXCL2 and TANs (Table IV).

Cumulative survival analysis. In univariate analysis of the 10-year CSR, the following parameters exhibited an association with the survival rate: Age, tumor size, body mass index, alcohol consumption, recurrence and neck dissection. Furthermore, the immunoexpression levels of *P. gingivalis*, CXCL2 and TANs were significantly associated with the 10-year CSR. Patients with a high immunoexpression of *P. gingivalis* had an increased risk with a lower 10-year CSR [hazard ratio (HR), 0.260; 95% CI, 0.135-0.503; P<0.001], and those with high immunoexpression levels of CXCL2 (HR, 0.283; 95% CI, 0.156-0.514; P<0.001) and TANs (HR, 0.494; 95% CI, 0.283-0.862; P=0.013) also had an enhanced risk with a lower 10-year CSR compared with patients with low immunoexpression levels of *P. gingivalis*, CXCL2 and TANs (Table V).

In the multivariate survival analysis of the 10-year CSR, the results demonstrated that recurrence, BMI, CXCL2 expression, *P. gingivalis* levels, TANs expression and alcohol consumption were independent risk factors for the prognosis of patients with OSCC (Table VI; Fig. 4).

Discussion

As aforementioned, there are a number of factors, including tobacco and alcohol use, as well as microbiological agents, that serve important roles in the progression of OSCC (20). Microorganisms are recognized as the primary risk factors for OSCC carcinogenesis (27), and the role of microorganisms in the promotion of OSCC has gradually become a novel area of research. An epidemiological study has demonstrated that 16-18% of carcinomas occur due to inflammation (28). Previous studies have also demonstrated that microbial pathogens, including Epstein-Barr virus, and hepatitis B and C virus, serve a role in tumor development (29-31).

Table III. Immunohistochemical expression of CXCL2 and TANs in 119 samples with strong staining for *Porphyromonas gingivalis* from patients with oral squamous cell carcinoma according to clinical data and follow-up.

	CXCL2			TANs		
Variable	Weak, n (%)	Strong, n (%)	P-value	Weak, n (%)	Strong, n (%)	P-value
Sex			0.631			0.824
Male	15 (50.0)	49 (55.1)		21 (55.3)	43 (53.1)	
Female	15 (50.0)	40 (44.9)		17 (44.7)	38 (46.9)	
Age, years			0.038[a]			0.229
<60	15 (50.0)	26 (29.2)		16 (42.1)	25 (30.9)	
≥60	15 (50.0)	63 (70.8)		22 (57.9)	56 (69.1)	
Survival status			0.003[a]			0.226
Alive	25 (83.3)	47 (52.8)		26 (68.4)	46 (56.8)	
Dead	5 (16.7)	42 (47.2)		12 (31.6)	35 (43.2)	
Differentiation			0.279			0.556
Well	20 (66.7)	48 (53.9)		24 (63.2)	44 (54.3)	
Moderate	8 (26.7)	25 (28.1)		10 (26.3)	23 (28.4)	
Poor	2 (6.7)	16 (18.0)		4 (10.5)	14 (17.3)	
Tobacco consumption			0.628			0.460
Yes	9 (30.0)	31 (34.8)		11 (28.9)	29 (35.8)	
No	21 (70.0)	58 (65.2)		27 (71.1)	52 (64.2)	
Alcohol consumption			0.189			0.158
Yes	7 (23.3)	17 (19.1)		5 (13.2)	19 (23.5)	
No	23 (76.7)	72 (80.9)		33 (86.8)	62 (76.5)	
T stage[b]			0.023[a]			0.015[a]
T1-2	21 (70.0)	41 (46.1)		26 (68.4)	36 (44.4)	
T3-4	9 (30.0)	48 (53.9)		12 (31.6)	45 (55.6)	
N stage[b]			0.091			0.053
N0	23 (76.7)	53 (59.6)		29 (76.3)	47 (58.0)	
N(+)	7 (23.3)	36 (40.4)		9 (23.7)	34 (42.0)	
Clinical stage			0.093			0.002[a]
I-II	16 (53.3)	32 (36.0)		23 (60.5)	25 (30.9)	
III-IV	14 (46.7)	57 (64.0)		15 (39.5)	56 (69.1)	
Recurrence			0.589			0.075
Yes	6 (20.0)	14 (15.7)		3 (7.9)	17 (21.0)	
No	24 (80.0)	75 (84.3)		35 (92.1)	64 (79.0)	
Periodontal condition			0.369			0.068
Well	11 (36.7)	41 (46.1)		12 (31.6)	40 (49.4)	
Poor	19 (63.3)	48 (53.9)		26 (68.4)	41 (50.6)	
Tumor size, cm			0.011[a]			0.141
<2.9	18 (60.0)	30 (33.7)		19 (50.0)	29 (35.8)	
≥2.9	12 (40.0)	59 (66.3)		19 (50.0)	52 (64.2)	
Treatment			<0.001[a]			0.848
None	3 (10.0)	8 (9.0)		4 (10.5)	7 (8.6)	
Surgery	11 (36.7)	30 (33.7)		13 (34.2)	28 (34.6)	
Chemotherapy + radiotherapy	0 (0.0)	10 (11.2)		2 (5.3)	8 (9.9)	
Comprehensive	16 (53.3)	41 (46.1)		19 (50.0)	38 (46.9)	

[a]P<0.05. [b]According to the 7th American Joint Committee on Cancer/Union for International Cancer Control staging system. P-values were determined using χ^2 or Fisher's exact tests. CXCL2, C-X-C motif chemokine ligand 2; TANs, tumor-associated neutrophils.

Furthermore, bacterial pathogens are associated with carcinogenesis with *H. pylori* infection, which is a causative factor in chronic gastritis, and aids in the development of gastric cancer (4).

Table IV. Association between *P. gingivalis* immunohistochemical expression and CXCL2 and TANs in 205 patients.

	CXCL2			TANs		
P. gingivalis	Weak, n (%)	Strong, n (%)	P-value	Weak, n (%)	Strong, n (%)	P-value
Weak	69 (80.2)	17 (19.8)	<0.001[a]	57 (66.3)	29 (33.7)	<0.001[a]
Strong	30 (25.5)	89 (74.8)		38 (31.9)	81 (68.1)	
Total	99 (48.3)	106 (51.7)		95 (46.3)	110 (53.7)	

[a]P<0.05. P-values were determined using χ^2 or Fisher's exact tests. CXCL2, C-X-C motif chemokine; TANs, tumor-associated neutrophils; *P. gingivalis*, *Porphyromonas gingivalis*.

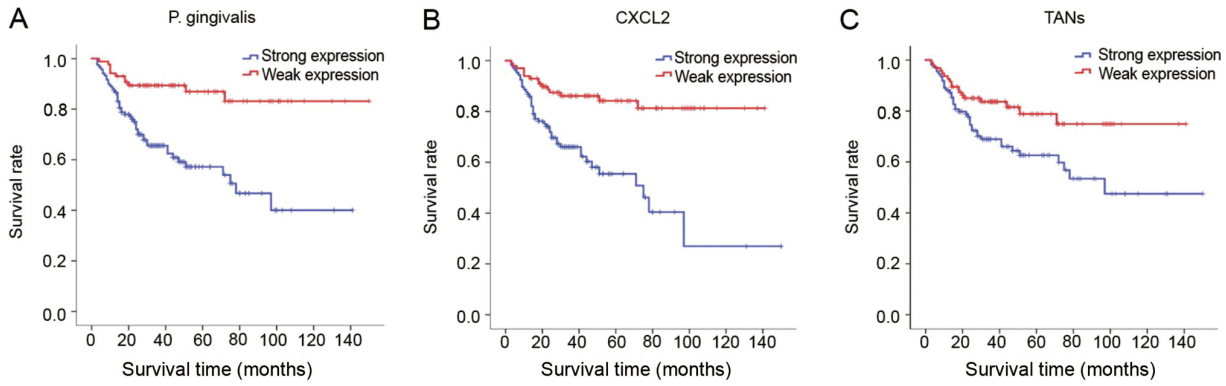

Figure 4. Survival curves for patients with oral squamous cell carcinoma according to *P. gingivalis*, CXCL2 and TANs immunoexpression levels. (A) Analysis of survival rate in patients with OSCC according to *P. gingivalis* expression. (B) Analysis of survival rate in patients with OSCC according to CXCL2 expression. (C) Analysis of survival rate in patients with OSCC according to TANs expression. *P. gingivalis*, *Porphyromonas gingivalis*; CXCL2, C-X-C motif chemokine ligand 2; TANs, tumor-associated neutrophils; OSCC, oral squamous cell carcinoma.

It has been reported that *P. gingivalis* is associated with the progression and metastasis of OSCC (32). *P. gingivalis* invades and exists in the neoplastic cells, reproduces and survives in the cytoplasm of infected cells, and spreads to the neighboring cells (33). In addition, following invasion, *P. gingivalis* evades the immune clearance mechanism of the host, meaning it survives, reproduces and affects the biological functions of immune cells (34). When *P. gingivalis* invades OSCC tissues, it is able to recruit myeloid-derived suppressor cells (MDSCs) by expressing factors, such as CXCL2 and IL-6 (35). Simultaneously, *P. gingivalis* promotes tumor progression by recruiting MDSCs by increasing the secretion of IL-6 and CXCL2 from infected oral dysplastic keratinocytes (35). Entry of microbial metabolites into the TME promotes tumor progression by eliciting tumor-potentiating immune cell responses (35).

In the present study, staining of *P. gingivalis* in OSCC samples was observed, and this was present to a lesser extent in the non-carcinoma samples. Furthermore, it was identified that staining was mainly localized in the cytoplasm of malignant cells. These findings indicated that, at the histological site, the bacteria have the ability to invade neoplastic cells *in vivo*. In addition, stronger *P. gingivalis* staining was identified to be associated with a poor prognosis.

Associations between oral cancer and tooth loss or periodontal disease have been reported in a previous study (36). IL-6 and IL-8 have been identified as vital cytokines involved in periodontitis under *P. gingivalis* infection (37). IL-6 has been verified as a biomarker to illustrate the role of *P. gingivalis* infection in favor of OSCC initiation and progression (38). IL-8 is commonly secreted in the TME, which promotes tumor progression via the chemotaxis of MDSCs (39). *P. gingivalis* expresses multiple types of virulence factors that serve different roles in subverting the host immune response (40). Different secreted virulence factors of *P. gingivalis* may exert contrasting influences on the production of IL-8 via various mechanisms (41). CXCL2 has been reported to promote the generation of monocytic MDSCs (42). Furthermore, CXCL5 and CCL5 are associated with tumor progression (43). The present results suggested that CXCL2 and TANs were markedly increased in OSCC tissues and the TME that were infected by *P. gingivalis*, indicating that *P. gingivalis* and CXCL2 are involved in the recruitment of TANs, which contributes to the progression of OSCC. However, using IHC, the association between the immunoexpression levels of related proteins in the TME of *P. gingivalis* infection and clinical data remains unclear, despite increased research that suggests a close association between *P. gingivalis* and OSCC (44).

In the present study, two mRNA microarray datasets were analyzed to identify DEGs. A total of 89 DEGs were identified between the two datasets, which included 22 downregulated genes and 67 upregulated genes. Higher mRNA expression levels of CXCL2 were associated with the TME of OSCC infected by *P. gingivalis*. Cancer cells with high CXCL2 expression due to transcriptional hyperactivation are primed for

Table V. Univariate analysis of Cox risk ratio model regression in 205 patients with oral squamous cell carcinoma.

Variable	No. of patients (%)	P-value	HR	HR (95% CI)
Sex		0.684	0.898	0.535-1.507
Male	103 (50.2)			
Female	102 (49.8)			
Age, years		0.023[a]	0.503	0.277-0.911
<60	76 (37.1)			
≥60	129 (62.9)			
Clinical stage		0.502	1.194	0.711-2.006
I-II	111 (54.1)			
III-IV	94 (45.9)			
Tumor size, cm		0.027[a]	0.820	0.069-1.097
<2.9	99 (48.3)			
≥2.9	106 (51.7)			
BMI		0.003[a]	0.453	0.270-0.761
<22.5	72 (35.1)			
≥22.5	133 (64.9)			
Tobacco consumption		0.941	1.022	0.579-1.803
No	147 (71.7)			
Yes	58 (28.3)			
Alcohol consumption		0.019[a]	0.511	0.231-1.134
No	169 (82.4)			
Yes	36 (17.6)			
Recurrence		<0.001[a]	0.801	0.587-1.939
No	175 (85.4)			
Yes	30 (14.6)			
T stage[b]		0.132	1.501	0.884-2.547
T1-2	135 (65.9)			
T3-4	70 (34.1)			
N stage[b]		0.847	1.059	0.594-1.888
N0	145 (70.7)			
N1-3	60 (29.3)			
Neck dissection		0.003[a]	2.206	0.495-0.858
None	88 (42.9)			
Yes	117 (57.1)			
Periodontal condition		0.636	1.133	0.676-1.898
Poor	101 (49.3)			
Well	104 (50.7)			
P. gingivalis		<0.001[a]	0.260	0.135-0.503
Weak	86 (42.0)			
Strong	119 (58.0)			
CXCL2		<0.001[a]	0.283	0.156-0.514
Weak	99 (48.3)			
Strong	106 (51.7)			
TANs		0.013[a]	0.494	0.283-0.862
Weak	95 (46.3)			
Strong	110 (53.7)			

[a]P<0.05. [b]According to the 7th American Joint Committee on Cancer/Union for International Cancer Control staging system. P-values were obtained using a Cox risk ratio regression model. CI, confidence interval; HR, hazard ratio; CXCL2, C-X-C motif chemokine; TANs, tumor-associated neutrophils; *P. gingivalis*, *Porphyromonas gingivalis*.

Table VI. Multivariate analysis using Cox risk ratio regression model in 205 patients with oral squamous cell carcinoma.

Variable	B	SE	Wald	P-value	HR	HR (95% CI) Upper	HR (95% CI) Lower
Age (<60 vs. ≥60 years)	0.510	0.361	1.994	0.158	0.600	0.296	1.219
Recurrence (no vs. yes)	1.370	0.328	17.446	<0.001[a]	0.254	0.134	0.483
BMI (<22.5 vs. ≥22.5)	0.665	0.311	4.574	0.030[a]	0.550	0.320	0.944
Tumor size (<2.9 vs. ≥ 2.9)	0.303	0.300	1.015	0.314	0.739	0.410	1.331
Neck dissection (no vs. yes)	0.093	0.174	0.287	0.592	0.911	0.648	1.281
CXCL2 (weak vs. strong)	0.854	0.362	5.549	0.018[a]	0.426	0.209	2.866
P. gingivalis (weak vs. strong)	0.688	0.399	2.975	0.035[a]	0.503	0.230	1.098
TANs (weak vs. strong)	0.688	0.310	0.549	0.039[a]	0.795	0.433	1.459
Alcohol consumption (no vs. yes)	0.134	0.063	4.494	0.034[a]	0.543	0.610	1.094

[a]P<0.05. P-values were obtained using a Cox risk ratio regression model. CI, confidence interval; HR, hazard ratio; CXCL2, C-X-C motif chemokine; TANs, tumor-associated neutrophils; *P. gingivalis*, *Porphyromonas gingivalis*

survival in the metastatic sites (14). CXCL2 expression appears to be involved in OSCC-induced bone destruction and promotes tumor progression (12). Furthermore, increased CXCL1 expression has been observed in different OSCC cell lines and tumor specimens, and is associated with leukocyte infiltration and lymph node metastasis (12). CXCL2 attracts $CD66b^+$ TANs into the tumor, producing chemokines that enhance cancer cell survival (45). Furthermore, the chemokine CXCL2 is the core cytokine that mediates lung metastasis and chemoresistance in breast cancer. When the CXCL2 receptor is blocked, the effects of chemotherapy against breast cancer and metastasis are effectively augmented (46). However, to the best of our knowledge, clinical research regarding the involvement of CXCL2 in promotion of cancer metastasis in OSCC has not been reported.

In recent years, the outlook on OSCC has changed and the tumor is no longer considered as a bulk of neoplastic cells, but rather as an entirety comprising a complex TME and neoplastic cells, and it has been suggested that tumor progression occurs via the TME and neoplastic cell interaction (47). The stromal component of the TME involves different cell types, such as TANs, cancer-associated fibroblasts and macrophages (47). In early tumors, TANs may be able to stimulate T cell responses (48), but in established tumors, TANs are immunosuppressive and associated with a more protumor phenotype with tumor progression (49). These subpopulation of cells interact with each other, as well as with neoplastic cells, via complex communication networks through secreted cytokines and chemokines (50). It has been reported that *H. pylori* is causally associated with the malignancies of gastric epithelia (51). *H. pylori* causes inflammation of the gastric mucosa by inducing gastric epithelial cells to secrete IL-8, resulting in the recruitment of inflammatory cells at the site of infection (52). Furthermore, IL-8, other chemokines and their receptors have been implicated in tumor development and metastatic progression. It has also been revealed that IL-8 enhances the generation of $CD163^+$ M2 macrophages, and $CD163^+$ macrophages exhibit a significant association with poor overall survival in patients with OSCC (53).

The present results suggested that, in OSCC, the TME infected with *P. gingivalis* exhibits increased CXCL2 secretion, which recruits TANs to the site of neoplastic cells and further promotes tumor development. It was demonstrated that the immunoexpression levels of CXCL2 were increased in patients with high expression levels of *P. gingivalis*. Another study with similar findings to the present study reported that *P. gingivalis* contributed to the accelerated secretion of CXCL8 and CCL2 (54). The number of $CD66b^+$ TANs in the TME of OSCC infected by *P. gingivalis* proportionally increased with the secretion of CXCL2, and the expression levels of *P. gingivalis* were associated with the expression levels of CXCL2 and TANs. Furthermore, all three of these factors were associated with the poor prognosis of patients. However, studies regarding the association between the TME of OSCC and *P. gingivalis* are considered to be at early stages, and future investigations are warranted to determine the underlying molecular mechanism.

In conclusion, to the best of our knowledge, to gain an increased understanding of the participation of *P. gingivalis* in the TME of OSCC, the association between the immunoexpression of *P. gingivalis* in OSCC and clinicopathological features and patient prognosis was analyzed for the first time in the present study. Using bioinformatics analysis, it was identified that CXCL2 was a DEG in the TME of OSCC infected by *P. gingivalis*. In addition, the associations of the immunoexpression levels of CXCL2 and TANs in OSCC with clinicopathological features and patient prognosis were further analyzed. The present study suggested a mutual interaction between *P. gingivalis* and the TME of OSCC, and that the course of chronic periodontal inflammation may create a tumor-favorable microenvironment that promotes tumor development and progression. Furthermore, strong immunoexpression levels of *P. gingivalis*, CXCL2 and TANs may be associated with a poor prognosis in patients with OSCC. Therefore, it is necessary to develop prevention strategies for patients with OSCC with chronic oral infection.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZCGo conceived and designed the study. ZCGu conducted the experiments. SJ, SLJ, XYJ and LLH performed the statistical analysis. ZCGu wrote the manuscript. ZCGu reviewed and edited the manuscript. All authors agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved. ZCGo and ZCGu confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Stomatological Hospital of Xinjiang Medical University (approval no. IACUC20180411-13; Urumqi, China). Written consent was obtained at the time of the initial data collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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