CCL18 overexpression predicts a worse prognosis in oral squamous cell carcinoma (OSCC)

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Oral squamous cell carcinoma (OSCC) presents severe morbidity and high mortality owing to local recurrence or remote metastasis. Molecular markers, including chemokines, might provide more efficient prognostic information or even therapeutic targets for the treatment of OSCC. Using quantitative RT-qPCR, we found that CCL18 was dramatically overexpressed in 30 OSCC tissues at the mRNA level in comparison with their adjacent non-cancerous oral mucosa tissues and 15 oral mucosa tissues from non-malignant patients. We then analyzed the relationship between CCL18 overexpression and patient clinical characters and outcomes using immunohistochemistry staining (IHC) in 102 paired OSCC cancerous and adjacent non-cancerous tissues; the increase in CCL18 expression was significantly higher in male patients (p=0.047), tumors of the palate and floor of the mouth (p=0.014), patients with positive lymph node metastasis (p=0.007), and patients with poor tumor differentiation (p=0.029). The median overall survival time and time-to-recurrence were 80.6 and 61.4 months in patients with high CCL18 expression, respectively, as against 93.4 and 81.6 months in patients with comparatively lower CCL18 expression, respectively (p=0.033 and 0.012, respectively; log-rank test). Multivariate analyses indicated age, poor differentiation, and CCL18 levels to be independent prognostic factors for predicting both overall and disease-free survival time. Our study suggests that CCL18 is a novel candidate marker for the OSCC malignancy and prognosis, including lymph node metastasis, time-to-recurrence, and disease-free survival time.

Key words: oral squamous cell carcinoma, biomarker, CCL18, survival, prognosis

Oral squamous cell carcinoma (OSCC) is the most common oral malignant tumor; it originates from the oral epithelium and develops into carcinoma *in situ*. According to statistics, more than 90% of oral malignant tumors are oral squamous cell carcinomas (OSCC) [1]. Globally, OSCC accounts for 2–4% of all cancers, and due to the lack of efficient early diagnosis and treatment methods, the 5-year survival rate is still only about 50%. In Asia, due to the betel nut chewing habits, OSCC has become one of the six most common malignant tumors, among lung, liver, stomach, colorectal, and prostate cancers [2]. Invasion and early metastasis of OSCC result in low 5-year survival rates, in spite of the advancement in surgery and chemo- and radiotherapy in the past decades. Hence, investigation and understanding of biomarkers in OSCC might contribute to further improvement in its diagnosis and treatment. However, the key molecular markers are yet to be identified.

The cancer immune microenvironment plays a key role in tumorigenesis and invasion [3, 4]. In addition to several types of immune cells, the cancer immune microenvironment involves a variety of chemokines. Chemokine ligand 18 (CCL18) belongs to the small cytokine family, and these are involved in immunoregulatory processes [5]. In tumor tissues, there seems to be a positive feedback loop in order to increase the CCL18 expression. A small amount of CCL18 can promote the differentiation and maturation of immature dendritic cells and the differentiation of the macrophage M2 subtype, which can further produce a large amount of CCL18. CCL18 ultimately plays an immunosuppressive role by promoting the differentiation of Treg cells and not T cells [4, 6]. Additionally, recent studies have also shown that CCL18 can contribute to the proliferation and invasion of OSCC through the AKT signaling pathway [7] and the mammalian target of rapamycin pathway (mTOR) [8].

Although CCL18 has promoted OSCC cell line proliferation and invasion *in vitro*, clinically there is still no prospective evidence to support the clear correlation between CCL18 and OSCC malignancy. In our study, after verification of CCL18 expression changes between normal oral mucosae and OSCC tissues using RT-PCR, we prospectively harvested samples of OSCC tissues and their corresponding neighboring non-cancerous tissues in 102 patients. Patients were constitutively followed up and all the tissues were immunostained to detect the level of CCL18. We found that CCL18 expression is strongly related to OSCC survival rate, lymph node metastasis, and tumor differentiation.

Patients and methods

Patients and samples. This study included a total of 147 patients (30 OSCC patients and 15 non-cancer patients for RT-qPCR study of the expression of CCL18; and 102 patients for paired IHC study of CCL18 expression). All patients underwent primary OSCC surgical resection or other non-cancer surgeries between May 2009 and May 2013 at Beijing Stomatological Hospital. The 132 OSCC patients had no previous radiotherapy and/or chemotherapy or other malignant diseases. Clinicopathological information including gender, age, tumor location, tumor grade, tumor (T) classification, node (N) classification, and tumor, node, and metastases (TNM) stage was obtained from clinical and pathological records. The TNM stages of patients were classified according to the 2012 American Joint Committee on Cancer Staging System. According to the guidelines of our hospital, surgical salvage is the first choice if the tumor is operable. Otherwise, patients will undergo salvage chemotherapy or chemoradiation therapy, which were excluded from this study. To detect CCL18 expression, fresh tissues were obtained from patients during the operation. A total of 102 OSCC cases with paired cancerous and non-cancerous peritumoral tissues were used as a prognosis study cohort whose tissues were immunohistochemically studied. A total of 15 patients with normal mucosae and an additional 30 OSCC cases with paired cancerous tissues and non-cancerous peritumoral tissues were used for quantitative RT-qPCR assays. These tissues were subjected to histological confirmation using frozen sections before quantitative RT-PCR assays and immunohistochemical studies.

Patient follow-ups were carried out by two physicians unaware of the study until June 2017. Complete follow-up data were available for all 102 cases, which served as a prognosis study cohort.

All participants in this study have signed written informed consent, and this investigation was performed in accordance with the Declaration of Helsinki and has been approved by the Ethics of Committee of Beijing Stomatological Hospital, Capital Medical University.

RNA extraction and quantitative RT-PCR (RT-qPCR). Tissue samples were dip frozen in liquid nitrogen and immedi-

ately used for RNA extraction. The tissue was homogenized using the MX-F Vortex mixer (SCILOGEX, LLC, USA). Total RNA was prepared from the frozen tissue samples using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA (500 ng) was then reverse-transcribed into cDNA using HiScript II Reverse Transcriptase (Vazyme, Nanjing, China). For RT-qPCR, the primers for CCL18 were as follows: CCL18 forward: CTGCCCAGCATCATGAAGG; CCL18 reverse: CCTCAGGCATTCAGCTTCAG. The primers for actin were as follows: actin forward: TGACGTGGACATCCGCAAAG; actin reverse: CTGGAAGGTGGACAGCGAGG

Data were represented as mean ± s.d. Actin was used as an internal control for comparison and normalization of the data. Assays were performed in triplicate using the Roche LightCycler 96 System.

Immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin overnight, then embedded in paraffin after a series of dehydration steps using gradient series of alcohol and transferred to xylene. Blocks of formalin-fixed, paraffin-embedded tissues were cut into 4 μm sections, deparaffinized with xylene, and rehydrated in a series of ethanol washes (100, 90, 80, and 70%). Slides were washed with phosphate-buffered saline (PBS) and treated with 3% H_2O_2 for 30 min to block endogenous peroxidase activity. Subsequently, sections were put into a water bath in 10 mM citrate buffer (pH 6.0) at 90°C to unmask epitopes. After antigen retrieval, sections were incubated with diluted anti-CCL18 primary antibody (NBP1-79940; Polyclonal; 1:300; NOVUS Biologicals, Littleton, CO, USA) or CD206 primary antibody (ab64693; Polyclonal; 1:2000; Abcam, Cambridge, UK) for 2 h followed by washing with PBS. Thereafter, corresponding biotin conjugated secondary antibodies were applied for 0.5 h. Horseradish peroxidase/streptavidin conjugate (PicTure-Plus kit; Zymed, South San Francisco, CA, USA) was then applied to the sections for 30 min followed by washing with PBS. Finally, the sections were incubated in diaminobenzidine (CCL18 staining, for 5 min) or 3-amino-9-ethylcarbozole (CD206 staining, for 10 min) to develop signals. A negative control, without the primary antibody, was run simultaneously. Positive immunostaining was defined as cytoplasmic and/or membrane immunoreactivity.

For CCL18, serial sections were examined under a light microscope (BH-2; Olympus, Japan) with a digital camera (DP72; Olympus). Images of low-power magnification (×200) fields were captured from each slide at random. Staining intensity was evaluated by mean optical density (MOD). The MODs of each image were counted and measured using Image-Pro Plus v6.0 software (Media Cybernetics Inc, Bethesda, MD, USA). All images were taken using the same microscope and camera set. For CD206 staining, the sections were examined under a light microscope (BH-2; Olympus, Japan) with a digital camera (DP72; Olympus). Images of interesting fields were captured from the slides.

Statistical analysis. The SPSS20.0 system was used for statistical analysis. For RT-PCR analysis, the differences in the CCL18 expression between 30 OSCC tissues, their corresponding peritumoral non-cancerous tissues, and 15 normal mucosa tissues were evaluated using the Wilcoxon-Matt-Whitney test. For immunohistochemistry analysis, CCL18 expression in 102 paired OSCC tissues and corresponding peritumoral non-cancerous tissues was half-quantified using MOD values (MOD_{ca} and MOD_{para}). The expression changes in CCL18 were represented as MOD_{incre} (MOD_{ca}/MOD_{para}). Associations between the expression changes of CCL18 (MOD_{incre}) and clinicopathological characteristics were evaluated using the chi-square test. The expression levels of CCL18 in all OSCC cases were separated into two groups as MOD_{IL} (comparatively low expression level) and MOD_{IH} (comparatively high expression level) according to the median MOD_{incre} value.

Overall survival (OS), disease-free survival (DFS), and TTR (time-to-recurrence) of patients were compared between the MOD_{IL} and MOD_{IH} groups according to the Kaplan-Meier method. Other potential clinical and histopathologic variables were also tested for prognostic significance evaluated using 3-year and 5-year OS and DFS rates. Overall survival was measured from the date of surgery to the date of the last follow-up examination or death, whereas DFS was measured from the date of surgery to the date on which recurrence and/or subsequent lymph node metastasis was first detected. Overall survival was defined as the interval between surgery and death or the last observation taken. The DFS was defined as the time between surgery and the date of any adverse event, whichever appeared first. Time-to-recurrence was defined as from the date of tumor resection to the detection of tumor recurrence. For the analysis of prognostic factors, differences in survival rates were evaluated using the log-rank test. Univariate and multivariate analyses of OS and DFS were performed using the Cox proportional hazards regression model. All statistics were 2-sided, and results were considered statistically significant at a probability of p<0.05.

Results

Clinicopathological characteristics and outcomes. The clinicopathologic characteristics of 102 OSCC patients whose specimens were used for IHC analysis are listed in Table 1. There were 54 males and 48 females, with a median age of 60 years (range, 33–83 years). The rate of pathological regional lymph node metastasis at the time of surgery was 36.3%. Among all 102 OSCC tissues, 72, 20, and 10 cases were well, moderately, and poorly differentiated, respectively (according to Broder's classification). At the time of the last follow up, 73 cases of the 102 patients (71.6%) were alive, 26 cases (25.5%) had died of OSCC, and 3 cases (2.9%) had died of other causes. Overall, 41 patients developed recurrent diseases, including 16 local recurrences (39.0%), 21 subsequent regional lymph node metastases (51.2%), and 6 distant recurrences (14.6%). The mean observation time was 61 months (range, 5–104 months).

Increase in CCL18 expression in OSCC. First, CCL18 expression was analyzed by RT-qPCR in 30 paired OSCC tissues and their adjacent non-cancerous oral epithelium tissues and another 15 normal oral mucosa specimens, which were harvested from non-tumor patients. The RT-qPCR analysis results are shown in Figure 1. Here, CCL18 was abundantly expressed in cancerous tissues 1.8612 (0.3179, 4.4351), in comparison. CCL18 expression in adjacent non-cancerous tissues was 0.2305 (0.0788, 0.8788), and in normal oral tissues 0.3200 (0.2300, 0.6500). There were statically significant differences between these groups (normal-paratumor p=0.485, normal-tumor p=0.012, tumor-paratumor p=0.001).

Secondly, the paired IHC staining of tissues from 102 OSCC cases showed that CCL18 expression increased in all the OSCC tissues when compared to their corresponding non-cancerous tissues. CCL18 was mainly expressed in OSCC cells, and also expressed by some cells in the mesenchymal tissue surrounding OSCC (Figure 1, ×200 magnification). CD206 was mainly expressed in mesenchymal tissue (Figure S1).

Table 1. Patient and tumor characteristics (n=102).

| Variable		No. of patients	%
Age, y	<60	51	50.0
	≥60	51	50.0
Gender	Female	48	47.1
	Male	54	52.9
Tumor location	Tongue	53	51.9
	Palate	7	6.9
	Buccal	9	8.8
	Floor of mouth	13	12.7
	Gingival	20	19.6
Pathologic T classification	pT1	37	36.2
	pT2	51	50.0
	pT3	8	7.8
	pT4	6	5.9
Pathologic lymph node metastasis	Negative (pN0)	65	63.7
	Positive (pN1-pN3)	37	36.3
TNM tumor stage	I	28	27.5
	II	30	29.4
	III	32	31.4
	IV	12	11.8
Tumor differentiation	Well	72	70.6
	Moderate	20	19.6
	Poor	10	9.8
Vital status at follow-up	Alive	73	71.6
	Death from OSCC	26	25.5
	Death from other than OSCC	3	2.9

Abbreviation: OSCC, oral squamous cell carcinoma.
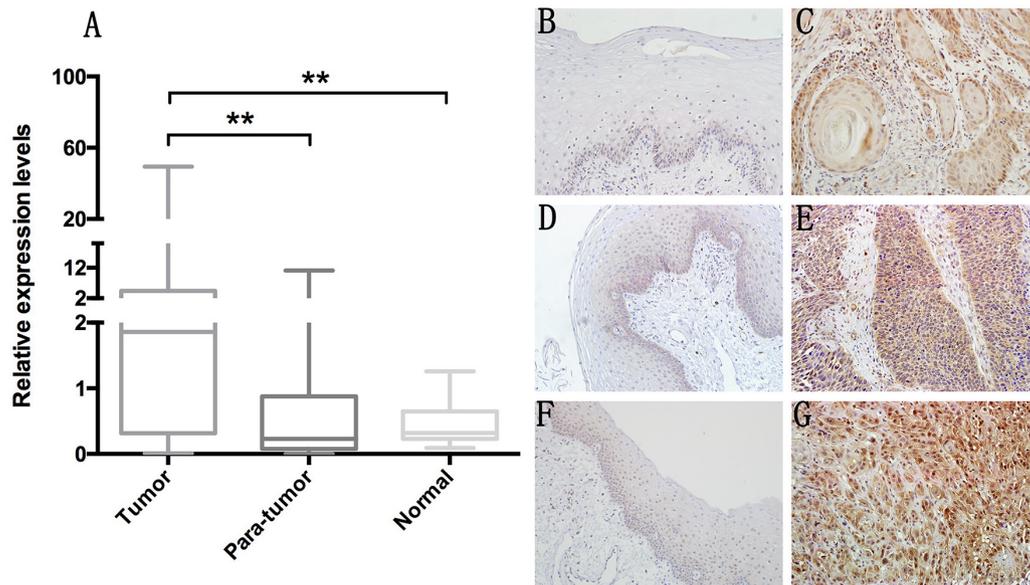
Figure 1. CCL18 expression in OSCC tumor, para-tumor, and normal oral mucosae. A) The mRNA expression levels of CCL18 were evaluated by qRT-PCR in 30-paired tumor and para-tumor tissues of OSCC patients and in 10 normal oral mucosa specimens, which were harvested from non-malignant patients. Results were normalized against the expression level of β-actin mRNA in each sample and then analyzed by Mann-Whitney U-test, **p<0.05, box plots display the median, 25th and 75th percentiles. It showed that CCL18 was abundantly expressed in the cancerous tissues 1.8612 (0.3179, 4.4351), in comparison, CCL18 expression in adjacent non-cancerous tissues was 0.2305 (0.0788, 0.8788), and in the normal oral tissues 0.3200 (0.2300, 0.6500). There were statically significant differences between these groups (normal-paratumor p=0.485, normal-tumor p=0.012, tumor-paratumor p=0.001). B, D, F) Stratified squamous epithelium of the peritumoral non-cancerous tissues. C, E, G) The corresponding OSCC tissues presented as the well, moderately and poorly differentiated subtypes. It showed that CCL18 expression in OSCC tissues obviously increased compared to their corresponding para-tumor tissues; and the expression levels were also augmented as the differentiation grade worsened. The magnification of the figures was 200×.

Association between increased CCL18 expression and clinicopathological characteristics. As in PCR assays, we found in IHC analysis that CCL18 expression was also substantially increased in OSCC tissues. We used MOD_{inc} (MOD_{ca}/MOD_{para}) to represent the changes of expression. By a median value, MOD_{inc} values of each case were separated into two groups as MOD_{IH} (comparatively high) and MOD_{IL} (comparatively low), representing the highly increased group and the moderately increased group respectively. Associations between increased expression of CCL18 and clinicopathological characteristics are summarized in Table 2. Shortly, greater increase in CCL18 expression was found in males (p=0.047), patients with tumors on the palate and floor of the mouth (p=0.014), and patients with positive lymph node metastasis (p=0.007) and poor tumor differentiation (p=0.029).

Association between increased CCL18 expression and OS, TTR, and DFS. The OS, TTR, and DFS curves were depicted by the Kaplan–Meier analysis using SPSS 20.0 software. Results showed that the mean OS time for MOD_{IH} patients was 80.6 months when compared with 93.4 months for MOD_{IL} patients (p=0.033, log-rank test; Figure 2A). The mean TTR for MOD_{IH} patients was 61.4 months, while that for MOD_{IL} patients was 81.6 months (p=0.012, log-rank test; Figure 2B). The mean DFS time for MOD_{IH} patients was

73.5 months, while that for MOD_{IL} patients was 91.4 months (p=0.024, log-rank test; Figure 2C).

Analysis of prognostic factors on OS. The 3-year and 5-year OS rates of the entire patient population were 82.1% and 76.8%, respectively. Univariate analysis of the OS rate among each variable as determined by the log-rank test is listed in Table 3. The cumulative overall survival rate of patients with comparatively increased CCL18 was significantly worse than for patients with other covariates (p=0.033). The following variables were also found to be associated with poor prognosis: age (p=0.005), poor differentiation (p=0.008). The univariate and multivariate analysis of OS using the Cox proportional hazards regression model is summarized in Table S1. Of the univariate analysis, age (p=0.009), poor differentiation (p=0.012), and high CCL18 expression (p=0.040) were found to be statistically significant prognostic factors affecting OS. Multivariate analysis of these 3 prognostic factors revealed that age (p=0.005), poor differentiation (p=0.035), high CCL18 expression (p=0.035) are independent prognostic factors for OS. Overall, these data indicate that high CCL18 expression is a statistically significant prognostic factor for OS in patients with OSCC.

Analysis of prognostic factors on DFS. The 3-year and 5-year DFS rates of the entire patient population were 75.8% and 71.4%, respectively. Univariate analysis of DFS among
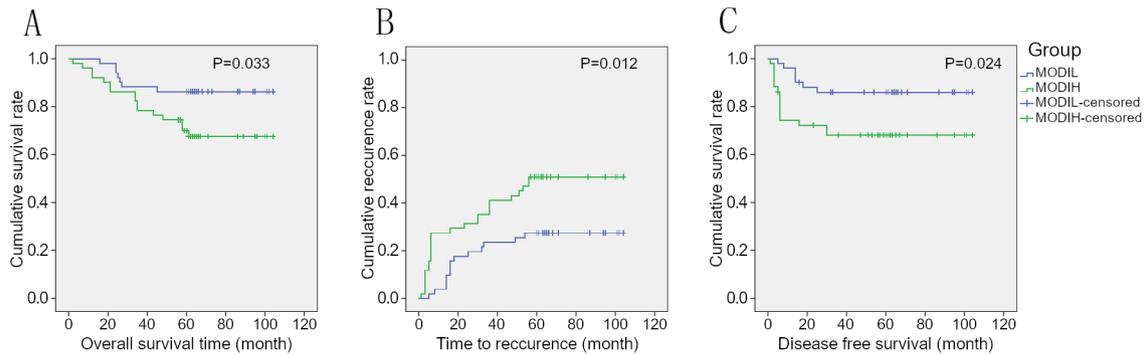
Figure 2. Association between increased CCL18 expression and OS, TTR, DFS. A) Correlation between increased CCL18 expression and the overall survival rate in patients with oral squamous cell carcinoma, as determined by the Kaplan-Meier method. The overall survival rate of patients who had comparatively high expression levels of CCL18 was statistically worse than that of patients with low expression. MOD_{IH} (n=51, mean = 80.6 months), MOD_{IL} (n=51, mean = 93.4 months), p=0.033, log-rank test. **B)** Correlation between increased CCL18 expression and the time to recurrence rate in patients with oral squamous cell carcinoma, as determined by the Kaplan-Meier method. The recurrence rate of patients with comparatively high expression level of CCL18 was statistically high than that of patients with low expression, MOD_{IH} (n=51, mean = 61.4 months), MOD_{IL} (n=51, mean = 81.6 months), p=0.012, log-rank test. **C)** Correlation between increased CCL18 expression and the disease-free survival rate in patients with oral squamous cell carcinoma, as determined by the Kaplan-Meier method. The disease-free survival rate of patients with comparatively high expression levels of CCL18 was statistically worse than that of patients with low expression. MOD_{IH} (n=51, mean = 73.5 months), MOD_{IL} (n=51, mean = 91.4 months), p=0.024, log-rank test.

Table 2. Association between CCL18 expression and clinicopathological factors.

Variable		No. of patients (%)		p-value
		Low (n=51)	High (n=51)	
Age, y	<60	24	27	0.552
	≥60	27	24	
Gender	Female	29	19	0.047 *
	Male	22	32	
Tumor location	Tongue	27	26	
	Palate	1	6	
	Buccal	5	4	0.014 *
	Floor of mouth	3	10	
	Gingival	15	5	
Pathologic T classification	pT1	19	18	
	pT2	26	25	0.065
	pT3	1	7	
	pT4	5	1	
Pathologic lymph node metastasis	Negative (pN0)	39	26	0.007 *
	Positive (pN1-pN3)	12	25	
TNM tumor stage	I	20	8	
	II	15	15	0.065
	III	11	21	
	IV	5	7	
Tumor differentiation	Well	39	33	
	Moderate	11	9	0.029 *
	Poor	1	9	

"low" and "high" represented the two groups with different expression levels of CCL18 classified by the median MOD value. *p-value was statistically significant by the chi-square test.

Table 3. Relationship between overall survival rate and clinicopathological variables.

Variable		3-year OS	5-year OS	CUM p-value
		%	%	
Age, y	<60	94.2	87.6	0.005*
	≥60	70	66	
Gender	Female	81.2	79.2	0.739
	Male	83.3	77.3	
Pathologic T classification	pT1 + pT2	86.4	79.3	0.154
	pT3 + pT4	64.3	55.5	
Pathologic lymph node metastasis	Negative (pN0)	84.6	82.2	0.084
	Positive (pN1-pN3)	81.1	66.1	
TNM tumor stage	I + II	84.2	82.1	0.190
	III + IV	82.2	69.7	
Tumor differentiation	Well + Moderate	87.5	83.3	0.008*
	Poor	73.3	59.8	
CCL18 expression	Low	88.2	85.7	0.033*
	High	78.4	67.5	

Abbreviation: OS, overall survival. * p-value was statistically significant by the log-rank test.

each variable as determined by the log-rank test is listed in Table 4. The DFS rates of patients that were older (p=0.006), with poor tumor differentiation (p=0.003), and MOD_{IH} (p=0.024) were significantly worse than that of patients with other covariates. The univariate and multivariate analyses of DFS by the Cox proportional hazards regression model are summarized in Table S2. In the univariate analysis, age (p=0.001), poor differentiation (p=0.006), and high CCL18 expression (p=0.032) were found to be statistically

significant prognostic factors for DFS. Multivariate analysis of the 3 prognostic factors revealed that high CCL18 expression (p=0.0118), age (p=0.005), and poor differentiation (p=0.014) were independent prognostic factors for DFS.

Discussion

This study demonstrated that CCL18 was highly expressed in poorly differentiated OSCC and OSCC patients with early lymphatic metastasis and poor survival rate. This suggests that CCL18 is related to OSCC prognosis. To date, there is no prospective clinical study examining the relationship between CCL18 expression and OSCC development. Our study suggests for the first time that CCL18 expression could be used as one of the prognostic indicators of OSCC.

In vitro experiments have shown that increased CCL18 in OSCC cells could accelerate their migration and invasion. This could be blocked by treatment with a neutralizing anti-CCL18 antibody or CCL18 knockdown [7]. In addition, exogenous CCL18 induced OSCC cell epithelial–mesenchymal transition (EMT), during which E-cadherin (epithelial marker) decreased and N-cadherin (mesenchymal marker) increased [8]. These studies support the results of our clinical study and suggest that CCL18 advances the epithelial cell proliferation and promotes EMT of OSCC, thereby promoting the growth and invasion of tumor tissue. In line with this, CCL18 is also related to the advancement of ovarian cancer [9] and non-small cell lung cancer [10] and deemed as a potential biomarker in ovarian cancer.

Besides its function directly in tumor cells, CCL18 is also one of the key mediators in the cancer immune microenvironment, according to some studies on cancers other than OSCC. Tumor-related CCL18 can activate macrophages and promote M2 differentiation [5, 11]. The M2 macrophages secrete a variety of factors accelerating the proliferation of local cells, providing a favorable microenvironment for tumor cell proliferation [12]. At the same time, M2 macrophages also release a variety of immunosuppressive factors, promote Treg differentiation and inhibit T helper T cell differentiation [13, 14]. Thus, Treg and M2 macrophages provide a favorable immune microenvironment for the proliferation and invasion of tumor cells [15, 16]. Nonetheless, there has been no study on CCL18's roles in the regulation of OSCC tumor immune microenvironment. Our study showed that CCL18 was expressed by OSCC cells and there were many CD206 (M2 macrophage marker) expressing macrophages surrounding the OSCC (Supplementary Figure 1), implying that CCL18 drives the advancement of OSCC through regulation of the immune microenvironment.

It has been demonstrated that CCL18 could promote cancer metastasis predominantly via binding to its receptor Nir1 (also named PITPNM3) in multiple human malignancies, including breast carcinoma [17, 18]. The PITPNM3-CCL18 binding induces Pyk2 and Src mediated signaling,

Table 4. Disease-free survival rate in relation to clinicopathological variables.

Variable		3-year DFS	5-year DFS	CUM p-value
		%	%	
Age, y	<60	86.9	84.2	0.01*
	≥60	64.6	58.5	
Gender	Female	77.8	75.0	0.68
	Male	72.9	65.8	
Pathologic T classification	pT1 + pT2	78.0	74.3	
	pT3 + pT4	54.5	44.4	
Pathologic lymph node metastasis	Negative (pN0)	81.0	78.0	0.09
	Positive (pN1-pN3)	65.7	58.6	
TNM tumor stage	I + II	81.1	78.3	0.21
	III + IV	68.3	60.6	
Tumor differentiation	Well + Moderate	83.3	80.7	<0.01*
	Poor	55.6	45.5	
CCL18 expression	Low	84.8	83.7	0.02*
	High	66.7	55.6	

Abbreviation: DFS, disease-free survival. *p-value was statistically significant using the log-rank test.

thus facilitating subsequent cancer cell metastasis [18]. Our results are in line with previous reports that show high CCL18 expression is related to lymphatic metastasis in OSCC patients.

Although lymph node metastasis often occurs during the early stage of OSCC, it is hard to detect by clinical examination or conventional imaging techniques [19]. Generally, neck dissection is the most reliable method by which to address lymph node metastasis within the neck, but this procedure can also result in a lymphatic leak, facial nerve injury, and dysfunction of the trapezius muscle. Therefore, indications for elective neck dissection in patients with clinically N0 OSCC remain controversial [20]. In our study, high CCL18 expression in tumor tissue was found to be a predictor of subsequent lymph node metastasis, thus could be used as an indicator for elective neck dissection. In accordance with this, CCL18 has been suggested as a factor of predicting lymphatic metastasis in non-small cell lung cancer [21] and ovarian cancer [9].

In this study, the results of RT-qPCR demonstrated that normal oral mucosa harvested from non-malignant patients expressed CCL18 far lower than the OSCC tissue and its neighboring non-cancerous tissue. This finding is consistent with observations in a previous study on colorectal cancer patients [22]. These data suggest that CCL18 might be used as a marker for early diagnosis of OSCC.

In summary, our study suggests that CCL18 expression is a common phenomenon in OSCC tissue and expression level correlated with tumor differentiation, metastasis, and prognosis. To our knowledge, prior to this investigation, there were only *in vitro* and *in vivo* studies regarding CCL18

and OSCC [7]. Our study provides the clinical evidence that CCL18 is closely related to the survival rate of OSCC, which is in accordance with previous studies. We demonstrate that CCL18 expression is strongly and negatively related to OS and DFS of OSCC patients and could potentially be used as a novel independent prognostic predictor of OSCC.

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CCL18 overexpression predicts a worse prognosis in oral squamous cell carcinoma (OSCC)

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Supplementary Information
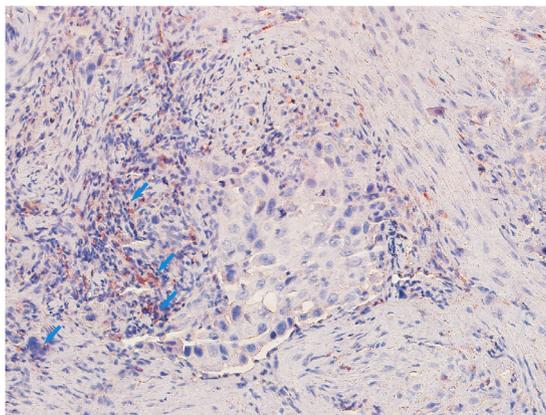
Suppl. Figure S1. CD206 expression in the interstitial tissue among the invading OSCC. It showed that CD206 (red staining) was expressed by many dense cells between the invading frontline OSCC cancerous epithelial cells. These CD206 expressing cells had smaller and rounder nuclei than other cells and some had large merged nuclei, which indicated that they are macrophages (blue arrows). Magnification 200×

Table S1. Univariate and multivariate analysis of overall survival in OSCC.

Variables		No. of patients (%)		Univariate Analysis			Multivariate Analysis		
		Alive	Dead	HR	95%CI	p-value	HR	95%CI	p-value
Age, y	<60	45 (57.7)	6 (25.0)	1		0.009*	1		0.005*
	≥60	33 (42.3)	18 (75.0)	3.459	1.362–8.784		3.83	1.497–9.798	
Gender	Female	37 (47.4)	11 (45.8)	1		0.74			
	Male	41 (52.6)	13 (54.1)	1.15	0.504–2.623				
Pathologic T classification	T1 or T2	69 (88.5)	19 (79.2)	1		0.164			
	T3 or T4	9 (11.5)	5 (20.8)	2.024	0.750–5.462				
Pathologic lymph node metastasis	Negative (pN0)	54 (69.2)	11 (45.8)	1		0.092			
	Positive (pN1-pN3)	24 (30.8)	13 (54.1)	2.023	0.892–4.588				
TNM tumor stage	I or II	47 (60.3)	10 (41.7)	1		0.197			
	III or IV	31 (39.7)	14 (58.3)	1.721	0.754–3.929				
Tumor differentiation	Well or Moderate	60 (76.9)	12 (50.0)	1		0.012*	1		0.035*
	Poor	18 (23.1)	12 (50.0)	2.859	1.261–6.483		2.433	1.065–5.557	
CCL18 expression	Low	44 (55.7)	8 (33.3)	1		0.04*	1		0.035*
	High	35 (44.3)	16 (66.7)	2.538	1.043–6.175		2.641	1.072–6.507	

Abbreviations: 95% CI, 95% confidence interval; HR, hazards ratio; OSCC, oral squamous cell carcinoma. *p-value was statistically significant using the Cox proportional hazards regression model.

Table S2. Univariate and multivariate analysis of disease-free survival in OSCC

Variables		No. of Patients (%)		Univariate Analysis			Multivariate Analysis	
		Disease-free	Disease	HR	95%CI	p-value	HR	95%CI
Age, y	<60	36 (58.1)	15 (37.5)	1		0.011*	1	
	≥60	26 (41.9)	25 (62.5)	3.36	1.324–8.527		3.831	1.497–9.806
Gender	Female	32 (51.6)	16 (40.0)	1		0.681		
	Male	30 (48.4)	24 (60.0)	1.189	0.521–2.712			
Pathologic T classification	T1 orT2	57 (91.9)	31 (77.5)	1		0.258		
	T3 orT4	5 (8.1)	9 (22.5)	1.772	0.658–4.776			
Pathologic lymph node metastasis	Negative (pN0)	40 (64.5)	25 (62.5)	1		0.105		
	Positive (pN1-pN3)	22 (35.5)	15 (37.5)	1.969	0.869–4.462			
TNM tumor stage	I or II	37 (59.7)	20 (50.0)	1		0.224	1	
	III or IV	25 (40.3)	20 (50.0)	1.669	0.731–3.806			
Tumor differentiation	Well or Moderate	50 (80.6)	22 (55.0)	1		0.006*	1	
	Poor	12 (19.4)	18 (45.0)	3.14	1.382–7.136		2.809	1.232–6.403
CCL18 expression	Low	37 (59.7)	14 (35.0)	1		0.032*	1	
	High	25 (40.3)	26 (65.0)	2.642	1.086–6.426		2.961	1.205–7.279

Abbreviations: 95% CI, 95% confidence interval; HR, hazards ratio; OSCC, oral squamous cell carcinoma. *p-value was statistically significant using the Cox proportional hazards regression model.