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Original Article

Correlations of abnormally upregulated CC chemokine ligand 18 (CCL18) with clinical stage and cervical lymph node metastasis status in serum and tumor tissue of patients with oral squamous cell carcinoma

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Abstract: This study aims to investigate the expression and clinical significance of CC chemokine ligand 18 (CCL18) in the serum and tumor tissue of the patients with oral squamous cell carcinoma (OSCC). Enzyme linked immunosorbent assay (ELISA) was used to detect the serum CCL18 contents in 59 patients with OSCC and 17 healthy adults; immunohistochemistry was used to detect the CCL18 expressions in 97 specimens of OSCC, 92 para-cancer tissue specimens, and 20 specimens of normal oral mucosa; and the correlations of the CCL18 expressions in the serum and tumor tissue of the OSCC patients with their clinicopathological parameters were then analyzed. The serum CCL18 expression in the OSCC patients was significantly higher than healthy people ($P < 0.05$), and that in the patients with advanced clinical stage was higher than those with early clinical stage ($P < 0.05$). The CCL18 expression in the tumor tissue of the OSCC patients was higher than those in para-tumor tissue ($P < 0.01$) and in normal oral mucosa ($P < 0.01$), and the expression in the patients with advanced clinical stage or cervical lymph node metastasis was higher than those in early clinical stage ($P < 0.05$) or without cervical lymph node metastasis ($P < 0.05$). CCL18 was abnormally upregulated in the serum and tumor tissue of the OSCC patients and closely related with the clinical stage and cervical lymph node metastasis status, suggesting that CCL18 might play an important role in the occurrence and development of OSCC.

Keywords: CCL18, oral squamous cell carcinoma, chemokines, ELISA, immunohistochemistry

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant head-neck cancer, with strong local invasion ability and high cervical metastasis rate. After such comprehensive series treatments with surgery as the main and radiotherapy and chemotherapy as the assistance, the 5-year survival rate ranges from 50% to 70%, and about one third of the patients would appear local recurrence and (or) distant metastasis [1-3]. Once the cervical metastasis appears, the patients' 5-year survival rate would be reduced by about 50% [4]. Because OSCC is located near important organs (such as brain, upper respiratory tract, or digestive tract), the wide-range surgical resection would not only impact the patients' facial form but also lead to great obstacles to language, chewing, swallowing, breathing, facial expressions, and other functional activities, thus seriously affecting the patients' quality of life. Therefore, further clarifying the pathogenic mechanism of the occurrence and development of OSCC and finding effective molecular markers or therapeutic targets would undoubtedly help the clinical idea of "early discovery, early diagnosis, and early treatment", thus exhibiting very important practical significance in improving the survival and life quality of the patients with OSCC.

Tumor microenvironment plays a key role in the occurrence and development of tumors, and inflammatory cytokines and their receptors are the important parts of the tumor microenvironment [5-7]. Study had shown that CC chemo-

kine ligand 18 (CCL18) played an important role in inflammations and immune responses [8], and its biological behaviors were closely related to the occurrence and development of tumors, which could not only inhibit but also promote tumors, and these roles had been confirmed in breast cancer [9], ovarian cancer [10], or prostate cancer [11]. Schrŏttner et al. [12] found that the serum CCL18 in the patients with B-Cell chronic lymphocytic leukemia (B-CLL) was significantly increased, while the low expression of CCL18 was the sign of good prognosis in these B-CLL patients. Hou et al. [13] reported that the plasma CCL18 level in the patients with GC was significantly higher than normal people, and the CCL18 concentration in the peripheral blood of GC was reduced with the increasing of the metastatic lymph node staging and surgical pathological staging and the decreasing of the tumor grading; the CCL18 level in the peripheral blood could reflect the progression of GC, thus helping the preoperative evaluation and prognostic judgment of GC patients. However, Nariţa et al. [14] reported that the serum CCL18 level in the late stage (III-IV) of Breast Cancer was higher than the early stage (0-II).

Currently, there is rare report about the expression of CCL18 in patients with OSCC. This study used ELISA and immunohistochemistry to investigate the expressions of CCL18 in the serum and tumor tissue of OSCC, respectively, and analyzed its correlations with the clinicopathological parameters, aiming to identify the roles of CCL18 in the occurrence and development of OSCC, and then providing new experimental evidence for the early diagnosis and treatment of OSCC.

Materials and methods

Patient source and specimen collection

59 serum specimens, 97 tumor tissue specimens, 92 para-tumor tissue specimens, and 20 normal oral mucosa specimens were collected from the OSCC patients admitted in the Department of Oral and Maxillofacial Surgery, Xiangya Hospital, Central South University from February 2009 to December 2011. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Central South University. Written informed consent was obtained from all participants. The OSCC tissue specimens and the para-tumor tissue specimens were obtained from tumor radical resection, and the para-tumor tissue specimen was the oral mucosa 1 cm outside the edge of the tumor tissue; the normal oral mucosa was sampled from the patients hospitalized for non-malignant tumor surgery during the same period. The serum of 17 healthy normal adults was taken from the physical examination Center, Xiangya Hospital, in December 2011. The collection of all the specimens was approved by the ethics committee of Xiangya Hospital. All the study subjects had not been performed preoperative chemotherapy, radiation, or other biological therapy, and the diseases that would impact the immune functions were excluded, such as diabetes, liver disease, tumor in other part, rheumatism, or rheumatoid. The primary tumor lesions and the cervical lymph node metastases were confirmed by the Department of Pathology, Xiangya Hospital.

The 59 OSCC serum specimens were collected from 50 males and 9 females, aging 31 to 71 years (mean age, 50.12 years). According to the differentiation criteria of tumors issued by WHO in 1971, these specimens were divided into 55 well-differentiated specimens, three moderately-differentiated specimens, and one poorly-differentiated specimen. According to the TNM staging criteria issued by the Union for International Cancer Control (UICC) in 2002, these specimens were divided into: 21 cases in T1 stage, 30 cases in T2 stage, 8 cases in T3 stage, and 0 case in T4 stage; 43 cases in N0 stage, 7 cases in N1 stage, and 9 cases in N2 stage; 59 cases in M0 stage and 0 case in M1 stage. According to the clinical staging: 16 cases in stage I, 26 cases in stage II, 8 cases in stage III, and 9 cases in stage IV. The 17 serum specimens from healthy normal adults were collected from 9 males and 8 females, aging 23 to 61 years (mean age, 42.12 years). The 20 normal oral mucosa specimens were collected from 14 males and 6 females, aging 27 to 56 years (mean age, 44.32 years); the 97 OSCC tissue specimens were collected from 79 males and 18 females, aging 27 to 79 years (mean age, 52.13 years), among which 82 were well-differentiated, 12 were moderately-differentiated, and three were poorly-differentiated; according to the TNM staging criteria: 28 cases

Table 1. Comparison of serum CCLI8 levels between the OSCC patients and the healthy adults (ng/L)

Group	Minimum	Maximum	Median	Average rank	Z	*P*
OSCC (n = 59)	73.22	423.61	151.66	41.19	-1.982	< 0.05
Healthy adults (n = 17)	92.58	207.19	134.21	29.15		

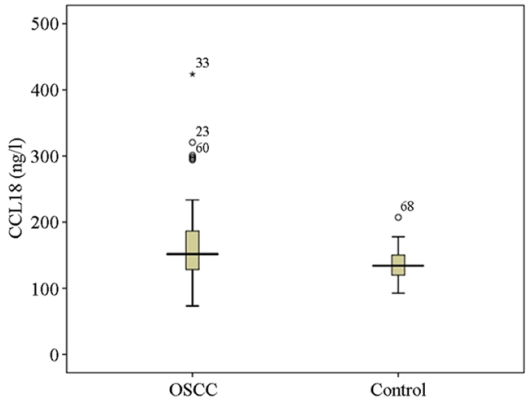

Figure 1. Comparison of serum CCLI8 levels between the OSCC patients and the healthy adults. (Three OSCC serum specimens appeared abnormal values, No 23, 33, and 60; one healthy serum specimen appeared abnormal value, No 68).

in T1 stage, 50 cases in T2 stage, 13 cases in T3 stage, and 6 cases in T4 stage; 62 cases in N0 stage, 26 cases in N1 stage, and 9 cases in N2 stage; 97 cases and 10 cases in M0 and M1 stages, respectively. According to the clinical staging: 22 cases in stage I, 34 cases in stage II, 20 cases in stage III, and 21 cases in stage IV.

Preparation of specimen

The fasting venous blood was sampled from all the study subjects in the morning (the specimens of the experimental group were sampled on the second day of admission); after stood still at room temperature for 10~20 min, the samples were centrifuged at 3000 rpm × 20 min; the supernatant was then carefully collected (if sediment appeared during this operation, the sample should be centrifuged again), and then stored at -80°C.

After sampled, the tissue specimen was trimmed, cut, fixed in 10% formalin solution for 24 h, and paraffin-embedded. All tissue specimens were prepared two successive sections, with one for HE staining and the other for immunohistochemical assay.

ELISA assay

The ELISA dual-antibody sandwich method was used to detect the CCL18 content in the serum samples, and the specific operations referred to the kit instructions; each sample was re-detected three times together with the standard control and the blank control.

Hematoxylin-eosin (HE) staining

The paraffin specimens were performed conventional slicing, dewaxing, hydration, 8-min hematoxylin staining, 10 s differentiation with 1% HCl solution, 15-min rinsing and re-blue staining, 1-min 0.5% eosin staining, 5-min xylene hyalinization, and neutral gum-mounting. The results were then observed under light microscope.

Immunohistochemistry

The paraffin specimens were performed conventional slicing, dewaxing, hydration, endogenous peroxidase elimination by 3% H_2O_2, microwave antigen retrieval, and 2 h calf serum closure; then, the specimens were cultured with 50 μl of goat anti-human CCL18 polyclonal antibody (1:100) overnight at 4°C, followed by dropwisely adding 50 μl of HRP-labeled rabbit anti-goat IgG secondary antibody working solution and 30-min incubation at 37°C. DAB coloration. Hematoxylin re-staining, HCl-ethanol differentiation, saturated lithium carbonate re-blue staining, gradient alcohol dehydration, xylene hyalinization, and neutral resin mounting were performed in turn. PBS instead of the primary antibody was used for the negative control.

Determination of immunohistochemical assay: according to the ratio of the cells with positive expression/area, the results were divided into four levels: (-): no positive cell or positive cells < 10%, 0 point; (+): positive cells were 10%~25%, 1 point; (++): positive cells were 25%~75%, 2 points; (+++): positive cells > 75%, 3 points. According to the staining intensity, four levels could be scored as 0 point (no staining or only

Table 2. Comparison of serum CCL18 levels among the OSCC patients with different clinicopathological parameters (ng/L)

Clinicopathological parameter		Cases	Minimum-maximum	Median	Average rank	Z	*P*
Gender	M	50	73.22-320.52	153.35	30.72	-0.759	0.448
	F	9	85.83-423.61	125.72	26.00		
Age (years)	< 50	29	73.76-423.61	162.33	33.55	-1.562	0.118
	≥ 50	30	73.22-294.06	147.51	26.57		
Disease duration (months)	≤ 3	35	73.22-423.61	150.66	29.54	-0.247	0.805
	> 3	24	85.83-320.52	155.46	30.67		
OSF	Without	47	73.22-423.61	150.66	28.83	-1.036	0.300
	With	12	73.76-320.53	186.63	34.58		
Tumor size	T_1+T_2	51	73.22-423.61	149.77	28.94	-1.196	0.232
	T_3+T_4	8	129.52-228.59	165.99	36.75		
Clinical stage	I+II	42	73.22-301.04	146.12	26.93	-2.159	0.031
	III+IV	17	122.35-423.61	169.64	37.59		
Differentiation	High differentiation	55	73.22-423.61	151.92	30.67	-1.116	0.283
	Moderate-poor differentiation	4	122.35-159.26	132.05	20.75		
Lymph node metastasis	No	43	73.22-301.04	146.99	27.81	-1.603	0.109
	Yes	16	122.35-423.61	165.99	35.88		

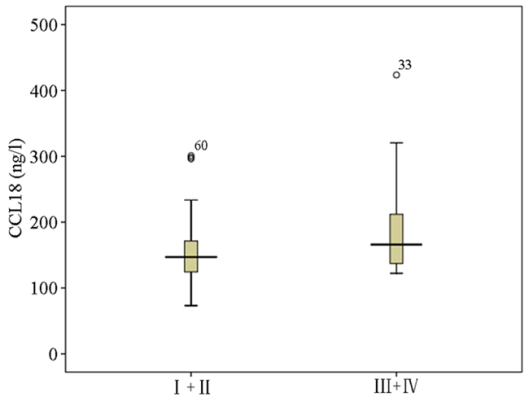

Figure 2. Comparison of serum CCL18 levels among the OSCC patients with different clinical stage (The I+II group had one abnormal value, No 60; the III+IV group had one abnormal value, No 33).

see nonspecific background staining), 1 point (pale yellow), 2 points (yellow), 3 points (buffy or brown). The immunohistochemical result was divided into four levels according to the sum of these two scores: negative (0 point), weakly positive (1 to 2 points), positive (3-4 points), and strongly positive (5-6 points).

Statistical analysis

SPSS19.0 statistical software was used for the statistical analysis, the counting data were performed the non-parametric Mann-Whitney U test or the Kruskal-Wallis H test, with $P < 0.05$ considered as statistically significant.

Results

Comparison of serum CCLI8 levels between the OSCC patients and the healthy adults

The minimum and maximum CCL18 contents in the 59 OSCC serum specimens were 73.22 ng/L and 423.61 ng/L with the median as 151.66 ng/L, and those in the 17 healthy serum specimens were 92.58 ng/L and 207.19 ng/L with the median as 134.21 ng/L (**Table 1**; **Figure 1**). The non-parametric Mann-Whitney U test revealed that the CCL18 level in the OSCC serum specimen was statistically significantly higher than that in the healthy serum specimen ($P < 0.05$).

Comparison of serum CCL18 levels among the OSCC patients with different clinicopathological parameters

Among the 59 OSCC patients (50 males and 9 females), the minimum and maximum serum CCL18 levels were 73.22 ng/L~320.5 ng/L and 85.83 ng/L~423.61 ng/L with the median as 153.35 ng/L and 125.72 ng/L; according to the median age, the patients were then divided into the < 50-year-old group (29 cases) and the ≥ 50-year-old group (30 cases), the minimum

Table 3. Comparison of CCL18expressions among OSCC tissue, para-OSCC tissue, and normal oral mucosa

Group	Expression intensity of CCL18					Z	*P*
	-	+	++	+++	Cases		
OSCC tissue	1	10	30	56	97	-6.017	0.000
Normal oral mucosa	4	13	2	1	20		
OSCC tissue	1	10	30	56	97	-3.510	0.000
Para-OSCC tissue	9	17	33	33	92		
Para-OSCC tissue	9	17	33	33	92	-4.069	0.000
Normal oral mucosa	4	13	2	1	20		

and maximum serum CCL18 levels were 73.76 ng/L~423.61 ng/L and 73.22 ng/L~294.06 ng/L with the median as 162.33 ng/L and 147.51 ng/L; according to the situations of smoking, drinking, and betel-chewing history, the OSCC patients were grouped and performed the non-parametric Mann-Whitney U test for the statistical analysis, and the results showed that the intergroup comparison of the serum CCL18 levels between the above two groups had no statistically significant difference ($P > 0.05$).

The OSCC patients were further grouped according to such clinicopathological parameters as disease course (≤ 3 months or > 3 months), with or without OSF, tumor size (T_1+T_2 and T_3+T_4), clinical stage (I+II and III+IV), tumor differentiation (high differentiation and moderate-poor differentiation), and lymph node metastasis situation to compare the serum CCL18 levels among different clinicopathological parameters. The results showed that the serum CCL18 levels showed statistically significant difference among different clinical stages ($P < 0.05$), and the CCL18 content was increased with the clinical stage increasing. The intergroup differences of the-parameter groups were not statistically significant ($P > 0.05$) (**Table 2**; **Figure 2**).

Expressions of CCL18 in OSCC tissue, para-OSCC tissue, and normal oral mucosa

Under high-power microscope, the positive expression of CCL18 appeared as yellow, buffy or brown staining mainly in the cytoplasm of OSCC cells and little on the cell membrane; CCL18 was mainly expressed in the intermediate-layer cells and inflammatory cells of para-tumor tissues; normal oral mucosa dose not express CCL18, the basal cells barely express it, and the intermediate-layer cells and inflammatory cells lightly express it. The CCL18 expression intensity descends in the following order: OSCC tissue, para-PSCC tissue, and normal oral mucosa, and the difference was statistically significant ($P < 0.05$). The pairwise comparison among the three groups revealed statistically significant differences between any two groups among the above three groups: OSCC vs normal oral mucosa ($Z = -6.017$, $P = 0.000$), OSCC vs para-OSCC ($Z = -3.510$, $P = 0.000$), and para-OSCC vs normal oral mucosa ($Z = -4.069$, $P = 0.000$) (**Table 3**; **Figure 3**).

Expressions of CCL18 in OSCC tissues with different clinicopathological parameters

The OSCC patients were further grouped according to such clinicopathological parameters as gender, age (median 53 years), betel-chewing history, tumor size (T_1+T_2 and T_3+T_4), clinical stage (I+II and III+IV), tumor differentiation (high differentiation and moderate-poor differentiation), and cervical lymph node metastasis situation to compare the intergroup difference in the CCL18 expression. The non-parametric statistical analysis revealed that the differences between early clinical stage (I+II) and late clinical stage (III+IV) ($Z = -0.821$, $P = 0.030$) and between the with- and without-lymph-node-metastasis groups ($Z = -2.28$, $P = 0.023$) were statistically significant. CCL18 was upregulated in the patients with late clinical stage and with lymph node metastasis than those with early clinical stage and without cervical lymph node metastasis. The comparisons between other clinicopathological-parameter groups showed no statistically significant difference ($P > 0.05$, **Table 4**).

Discussion

The occurrence and metastasis of tumors are closely related to their surrounding environments. Tumor microenvironment is the local internal environment co-composed by the infiltrative immune cells, stromal cells, active media secreted by them, and tumor cells. Inflammation modulators and cellular effector are important elements of the tumor microenvi-

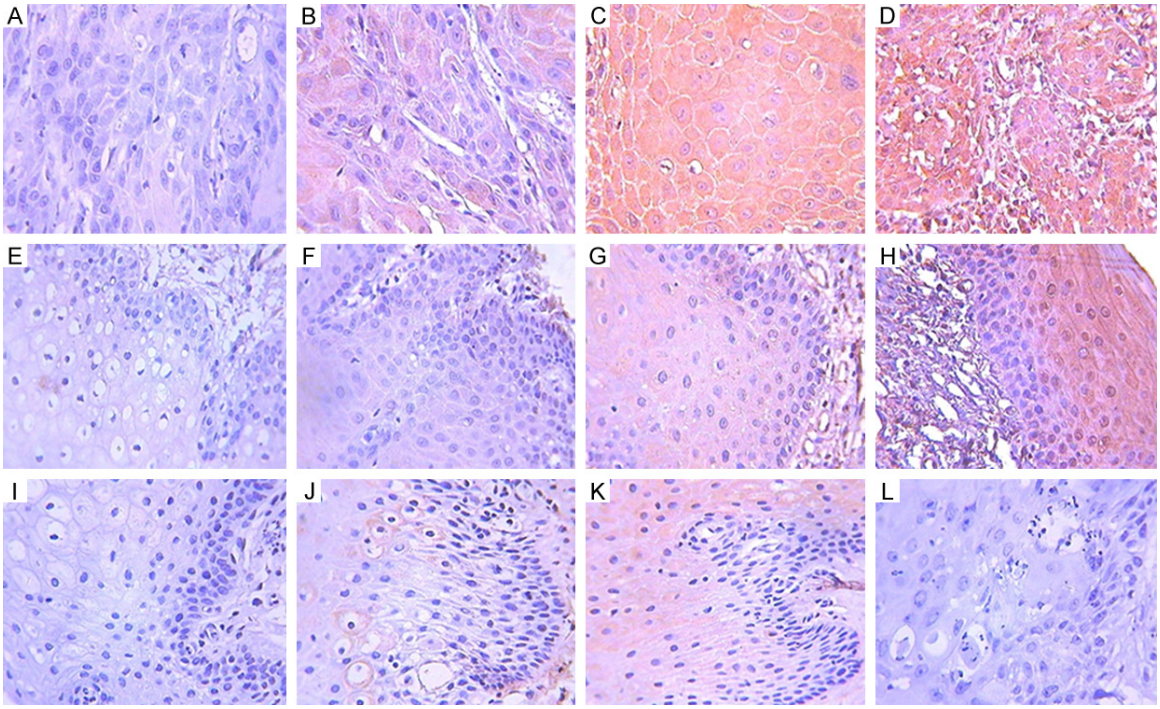

Figure 3. Expression detection of CCL18 by immunohistochemistry (PV, × 400). A-D: OSCC tissue: negative, weakly positive, positive, and strongly positive expressions of CCL18; E-H: Para-OSCC tissue: negative, weakly positive, positive, and strongly positive expressions of CCL18; I-K: Normal oral mucosa: negative, weakly positive, and positive expressions of CCL18; L: Negative control of OSCC tissue.

Table 4. Expressions of CCL18 in OSCC tissues with different clinicopathological parameters

Group		Expression intensity of CCL18					Z	*P*
		-	+	++	+++	Cases		
Gender	M	1	7	26	45	79	-0.084	0.933
	F	0	3	4	11	18		
Age (years)	< 53	1	4	13	27	45	-0.353	0.724
	≥ 53	0	6	17	29	52		
Betel-chewing history	No	0	4	19	33	56	-0.655	0.513
	Yes	1	6	11	23	41		
Tumor size	T_1+T_2	1	10	24	43	78	-1.356	0.175
	T_3+T_4	0	0	6	13	19		
Clinical stage	I+II	1	10	18	28	57	-2.166	0.030
	III+IV	0	0	12	28	40		
Differentiation	High	1	9	26	46	82	-0.821	0.412
	Moderate-poor	0	1	4	10	15		
Lymph node metastasis	No	1	9	21	31	62	-2.28	0.023
	Yes	0	1	9	25	35		

ronment, and the inflammations in tumor microenvironment could damage the in vivo immune system, alter the microenvironmental signal pathways, affect the normal stem cell niche, and promote tumorigenesis. The tumor development could also stimulate the production of the inflammatory microenvironment and further promote the proliferation of tumor cells. Chemokines are an important family constituting inflammations and immune media, and che-

mokines and their receptors are closely related to the malignant manifestations of tumor cells. On one hand, chemokines could chemotaxis the immune cells to infiltrate into tumors, thus inhibiting tumor's growth and metastasis by activating the host-tumor-specific immune response; on the other hand, chemokines could regulate the secretion of cytokines by autocrine or paracrine manner, regulate the immune system, chemotaxis the tumor cells, promote tumor-associated angiogenesis, digest the extracellular matrix, stimulate the growth of tumor cells, or increase the adhesion between tumor cell and extracellular matrix, thus directly or indirectly promoting tumor growth and metastasis [15-17]. CCL18 is a novel chemokine, mainly produced by M2 macrophages, and highly expressed in a variety of chronic inflammatory and fibrotic diseases [18], as well as in the body fluids (serum/plasma, ascites) and tumor tissue of the patients with such malignant rumors as leukemia [12], gastric cancer [19], ovarian cancer [10, 20, 21], breast cancer [9], or liver [22]; it might be involved in the biological behaviors of tumors, such as proliferation, invasion, adhesion, metastasis, and angiogenesis, so it is closely related to the occurrence and development of tumors, and it's an independent prognostic factor towards the occurrence and development of tumors.

This study found that the serum CCL18 in the OSCC patients was significantly higher than that in healthy adults, consistent with the results in leukemia, stomach cancer, ovarian cancer, or breast cancer. The statistical analysis towards the correlations of the serum CCL18 level with the clinicopathological parameters in 59 OSCC patients revealed that with the clinical stage increasing, the serum CCL18 content was also correspondingly increased, suggesting that CCL18 was an important tumor marker of OSCC and could be used for the auxiliary diagnosis of OSCC. The results was consistent with Nariţa et al. [14] who reported that the serum CCL18 level in the patients with late breast cancer (stage III-IV) was higher than that in the early stage (0-II). With the tumor development, the body's immune response would then be enhanced, and the numbers of immune cells and inflammatory cells were then significantly increased; therefore, the immune cells (macrophages and DC) and inflammatory cells-derived CCL18 would also be increased significantly to participate in the anti-tumor activity. In addition, in order to meet the growth needs of the tumor, the cells in the tumor microenvironment might also secrete large amounts of inflammatory cytokines, such as CCL18, to promote the tumor development.

CCL18 was significantly upregulated in the OSCC tissue than the para-OSCC tissue and normal oral mucosa, and it was also highly expressed in the para-OSCC tissue, suggesting that CCL18 might play an important role in the occurrence and development of OSCC. Study found that CCL18 could chemotaxis such immune cells and inflammatory cells as lymphocytes, immature DC, and monocytes, enhance the phagocytic functions of anti-inflammatory cells, and promote them to release the inflammatory mediators, thus directly participating in the inflammatory processes and the immune response in tumor tissues. Meanwhile, the activated immune cells and inflammatory cells could secrete large amounts of CCL18 to enhance their own functions, thus playing strong anti-tumor effects; on the other hand, with the development of tumors, the immune cells and tumor cells could secrete a large number of CCL18 so that such roles of CCL18 towards tumor tissues as proliferation, invasion, adhesion, metastasis, and angiogenesis would be activated, and the tumor would thus be promoted. Because of this active dual-role of CCL18 during the period of tumorigenesis, CCL18 was abnormally regulated in the OSCC tissue. The para-OSCC tissue, which located next to the forefront of tumor infiltration, might produce significant local inflammatory response due to the stimulations of tumor cells; therefore, a large number of inflammatory cells and immune cells might gather, participate in the tumor immune response, and provide the stromal cells needed by the tumor microenvironment. These inflammatory cells and stromal cells might secrete large amounts of CCL18 to enhance its cell functions and its local anti-inflammatory and pro-inflammatory effects, thus leading to the high expression of CCL18 in the para-OSCC tissue than that in the normal oral mucosa.

Our results showed that CCL18 was mainly expressed in the cytoplasm of OSCC cells but little expressed in the nuclei and on the mem-

brane, and it was also expressed in the stromal cells; in the para-OSCC tissue, CCl18 was mostly expressed in the cytoplasm of intermediate-layer stratified squamous epithelial cells, but rarely or not expressed in the base layer and epithelial layer. Furthermore, the oral mucosal region with inflammatory cell infiltration also showed the expression of CCL18. We speculated that in the OSCC tissue, CCL18 might be mainly secreted by the tumor cells while in the para-OSCC tissue and oral mucosa, CCL18 was mainly secreted by the inflammatory cells, consistent with Wang et al. [23] that CCL18 was mainly secreted by the ovarian tumor cells instead of the macrophages in the para-tumor tissue.

Chen [24] reported that the increased number of breast tumor-associated macrophages with positive CCL18 expression ($CCL18^+$ TAMs) in 562 breast cancer cases was related to the tumor size, clinical stage, histological grade, and cervical lymph node metastasis, and increased with the increasing of these clinicopathological parameters. TAMs could secrete CCL18, thus promoting the invasion and metastasis of breast cancer cells, so the more TAM in the breast cancer tissue that could secret CCL18, the higher chances of lymph node and organ metastasis in these breast cancer patients, and the worse prognosis would be. Intervening the secretion of CCL18 by TAMs could significantly inhibit the metastasis of these breast cancer cells. However, recently, Yuan et al. [25] reported that the high CCL18 expression in the macrophages, which were locally infiltrated by the colorectal tumor tissue, was the independent prognostic marker of higher survival rate in these patients. Our results showed that CCL18 was upregulated in the OSCC patients with late clinical stage and cervical lymph node metastasis than those with early clinical stage and without cervical lymph node metastasis, consistent with Chen. However, due to the dual roles of CCL18 towards tumors, whether the expression of CCL18 was the sign of poor prognosis in the OSCC patients and its exact regulatory mechanisms in the occurrence and development of OSCC still needed further in-depth researches.

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Disclosure of conflict of interest

None.

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