Chemokine (CC motif) ligand 18 upregulates Slug expression to promote stem-cell like features by activating the mammalian target of rapamycin pathway in oral squamous cell carcinoma

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Key words
Cancer stem(-like) cell (CSC), chemokine (CC motif) ligand 18 (CCL18), epithelial–mesenchymal transition (EMT), oral squamous cell carcinoma (OSCC), Slug

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Funding Information
National Natural Science Foundation of China (81371148, 81671000).

Received January 27, 2017; Revised May 26, 2017; Accepted May 30, 2017

Cancer Sci 108 (2017) 1584–1593
doi: 10.1111/cas.13289

Oral squamous cell carcinoma is the most common and aggressive epithelial tumor in the head and neck region, accounting for approximately 90% of oral malignancies and with a rising incidence in many countries. The 5-year survival rate of OSCC patients has not significantly improved and remains <50%, despite extensive studies on pathogenesis, diagnosis, and therapy.\(^1\)–\(^3\) Cervical lymph node metastasis and occasional distant organ metastasis are believed to be the leading causes of the high mortality of OSCC.\(^4\)–\(^6\) So, it is necessary to elucidate mechanisms underlying OSCC tumorigenesis and development.

Chemokine (CC motif) ligand 18 is one of important molecular components in immunological and inflammatory processes, triggering biological activity in dendritic cells, monocytes/macrophages, fibroblasts, and cancer cells. It is involved in the remodeling of the tumor microenvironment and has crucial roles in oncogenesis, invasiveness, and metastasis.\(^5\)–\(^6\) Recent studies have shown that increased expression of CCL18 was observed in a variety of cancers and associated with clinicopathological features.\(^7\)–\(^15\) We previously reported that CCL18 was overexpressed in primary OSCC tissues and associated with tumor TNM stage. Moreover, increased CCL18 acted in an autocrine manner to enhance cancer cells growth and invasion.\(^13\) However, the underlying molecular mechanisms by which CCL18 contributes to cancer cell invasion and metastasis in OSCC remain unclear.

Cancer stem(-like) cells are a subgroup of cancer cells with a small percentage and have an ability to regenerate various cell types within tumors. Notably, CSCs are considered more resistant to radiotherapy and chemotherapy and responsible for tumor maintenance, metastasis, and recurrence.\(^14\)–\(^15\) Cancer stem(-like) cells have been isolated from many cancer types, including head and neck squamous cell carcinoma.\(^15\) and
accumulating evidence has shown a correlation between EMT and the acquisition of stem-cell-like traits in neoplastic cell populations.\(^{(16)}\) Epithelial–mesenchymal transition is a strictly regulated process during embryonic development and cancer progression through which an epithelial cell loses its cell polarity and cell–cell adhesion, but acquires the capacity to migrate and metastasize.\(^{(17,18)}\) Many groups have confirmed the hypothesis that the induction of EMT promotes cancer cell migration and invasion, and contributes to the therapeutic resistance and enrichment of cells with stem-cell-like features.\(^{(15)}\)

In this study, for the first time, we evaluated the effects of exogenous CCL18 on EMT and stem-cell-like characteristics in OSCC cell lines, and then determined the relationship between CCL18 and CSC phenotypes in human OSCC specimens. Furthermore, we detected the potential mechanisms underlying the function of CCL18, and used Slug RNAi and mTOR inhibitor to reverse this function in vitro. Our results suggest that CCL18 promotes EMT and stem-cell-like traits of OSCC by activating the mTOR–Slug pathway, providing new targets for the early diagnosis and treatment of OSCC.

Materials and Methods

Cell lines and tumor samples. The OSCC cell lines, HSC6 and CAL33, were kindly provided by J. Silvio Gutkind (NIH, Bethesda, MD, USA). SCC15 was purchased from ATCC (Manassas, VA, USA). The cells were incubated in a mixture of DMEM (Gibco, Grand Island, NY, USA) and FBS (Gibco) at a ratio of 9:1, 5% CO\(_2\), 37°C.

A total of 45 OSCC patients who underwent surgical resection at the Department of Craniofacial Surgery, Guanghua School of Stomatology, Sun Yat-sen University (Guangzhou, China) were enrolled in the study. Primary OSCC tissues were obtained from the most representative areas of each case, postoperatively. Medical records for all OSCC cases were reviewed for clinical information. We obtained informed consent from each patient. The study was approved by the Ethics Committee of Guanghua School of Stomatology, Sun Yat-sen University.

Information regarding reagents and antibodies is listed in Table S1.

Small interfering RNA. Slug siRNA and Si-NC were designed by Ribobio (Guangzhou, China). Three different Slug siRNA duplexes were tested, and the sequences were as follows: SiSlug1, 5'-GGACACAGTGCTGCTGAA-3'; SiSlug2, 5'-GGAGCATACAGCCCATCA-3'; and SiSlug3, 5'-CTTCCAGGACACATTAGAA-3'. Cells were seeded in 6-well plates overnight and then transfected with siRNAs using the Lipofectamine RNAiMAX reagent and Opti-MEM (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction.

Immunohistochemistry. Tissue sections from paraffin-embedded OSCC were dewaxed in xylene and rehydrated in a graded alcohol series, soaked with 0.1% Triton X-100, incubated in 3% H\(_2\)O\(_2\) to eliminate endogenous peroxidase activity, heated in sodium citrate buffer (pH 6.0) for antigen retrieval, and then incubated in goat serum followed by incubation with CCL18 or Bmi-1 primary antibody overnight at 4°C. After washing with PBST, the sections were incubated with secondary antibody for 30 min, and then visualized with DAB solution and counterstained with hematoxylin. The expression of CCL18 was quantified using a visual grading system based on the degree of staining. The positive cell percentages were classified as: 0, <5%; 1, 5–30%; 2, 30–70%; and 3, >70%. Staining intensity was graded as: 0, none; 1, weak; 2, moderate; and 3, strong. Five representative fields at 400× magnification were evaluated for each sample. A weighted staining value (S) was calculated by multiplying the positive cells percentage and the score of the staining intensity. Finally, all samples were assigned to three levels according to the S value: negative, S = 0; low expression, 0 < S ≤ 6; high expression, 6 ≤ S ≤ 9. Bmi-1 expression was defined as positive when typical nuclear staining was observed.

Western blot analysis. The cultured cells were lysed with RIPA buffer (Abcam, Cambridge, MA, UK), then centrifuged, and the precipitate discarded. The total protein concentrations of the supernatant were measured by the BCA protein assay kit (Cwbio, Beijing, China). Then the proteins were separated by 10% or 12% SDS-PAGE and blotted onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. Subsequently, the membranes were washed with TBST and incubated with HRP-conjugated secondary antibody for 1 h. The immunoreacted bands were visualized with a highly sensitive chemiluminescence (ECL) detection system (Millipore). ImageJ (Bethesda, MD, USA) software was used to quantify the gray value and S value of the bands and the ratio (B) of the target protein’s A to internal reference protein’s A. Finally, the ratio (C) values of the test group’s B to the control group’s B from triplicate experiments were obtained for statistical analysis.

Reverse transcription–quantitative real-time PCR. Total RNA was prepared with TRizol (Invitrogen, Carlsbad, CA, USA) and reverse to synthesize cDNA according to the manufacturer’s procedure (TaKaRa, Shiga, Japan). The real-time PCR was carried out using the LightCycler 480 SYBR Green I Master system (Roche, Basel, Switzerland). The relative quantification of mRNA levels was evaluated by the comparative Ct (ΔΔCt) method with GAPDH as the internal control gene. The primers used were: for Slug, sense, 5'-TATTTTGTTGGT–TACGACAGG-3' and antisense, 5'-GACGCAATCAATGTT–TACTCG-3'; for OCT4, sense, 5'-GGTGATTCAGCCAAACGACCA-3' and antisense, 5'-CCTCTCACCTGGTCTCAGAT-3'; for Bmi-1, sense, 5'-CCAGGGGTCTTTCACAAATAGT-3' and antisense, 5'-CCGATCTACACATCTGTCT GTG-3'; and for GAPDH, sense, 5'-GCCGGTCAAGGCTGACAC-3' and antisense, 5'-TGTGAAGACGCCTAGGGA-3'.

Immunofluorescence. The prepared cells were plated on confocal culture dishes and cultured normally overnight. Cells were then fixed with 4% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 20 min, and blocked with goat serum for 30 min. Cells were treated with primary antibodies overnight at 4°C, followed by Dylight 594-conjugated and Dylight 488-conjugated secondary antibodies (1:200; Abcam) protected from light for 1 h at 37°C; subsequently, cell nuclei were stained with DAPI (Invitrogen) for 5 min. The confocal culture dishes were finally observed under a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) and representative fields of view at 200× magnification were randomly imaged for each group.

Transwell assay. Cell migration and invasion capacities were measured by a Transwell assay (Corning, Toledo, OH, USA). In contrast to the migration assay, the upper chamber of the insert was precoated with 0.1 mL (300 µg/mL) Matrigel matrix (Corning) for the invasion assay. In both assays, the prepared cells were seeded in the upper chamber with sernum-free medium, but the medium of the lower chamber was
supplemented with 10% FBS as a chemoattractant. After incubation for 24 h, the cells were fixed with 4% formaldehyde. The cells not migrating or invading through the pores were removed with a cotton swab. Those that had migrated or invaded onto the lower surface of membrane were stained by crystal violet. Finally, five representative fields at 100 × magnification were randomly imaged and quantified for each well using a light microscope (Carl Zeiss).

**Spheroid formation assay.** Cells were seeded in low-adhesion 6-well plates (2000 cells/well) and cultured in DMEM/F12 (Gibco) without FBS. This tumor sphere medium was supplemented with N2 supplement, 20 ng/mL human recombinant basic fibroblast growth factor, and 20 ng/mL epidermal growth factor (Gibco) in the absence or presence of CCL18 (20 ng/mL) and/or INK128 (100 µM). After 10 days of incubation, the primary spheres larger than 100 µm were counted for each well. Then the primary spheres were dissociated into single cells and seeded in the same culture conditions. Ten days later, secondary spheres larger than 100 µm were similarly counted.

**Flow cytometry.** Cells were digested by 0.25% trypsin and 0.02% EDTA (Gibco). After centrifuged in media, the cells were washed and counted in PBS containing 0.5% BSA. They were then adjusted to a concentration of 1 × 10⁶ cells/mL and incubated within the allophycocyanin-conjugated anti-human CD133 for 45 min, and finally washed. The ALDH enzymatic activity was measured with the ALDEFLUOR kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer’s protocol. The cells treated with ALDH inhibitor diethylaminobenzaldehyde (50 mmol/L) were used as a negative control. Flow cytometry analysis was carried out on CytoFLEX S (Beckman Coulter, Brea, CA, USA). Duplicates and dead cells were excluded by gating with forward scatter and side scatter.

**Statistical analysis.** All statistical analyses were undertaken with spss 20.0 software (SPSS, Chicago, IL, USA). Data were analyzed using Student’s t-test or one-way ANOVA and were displayed as the means ± SEM of at least three independent experiments. The association between Bmi-1-positive and CCL18-high expression in immunohistochemistry experiments was analyzed using the χ²-test. P-values < 0.05 were considered statistically significant.

**Results**

Chemokine (CC motif) ligand 18 (CCL18) promoted epithelial–mesenchymal transition in oral squamous cell carcinoma. (a,b) HSC-6 and CAL33 cells were cultured in the absence or presence of CCL18 (20 ng/mL) for 48 h, then their migration and invasion abilities were examined by Transwell assay. Representative pictures and the mean number of cells that migrated (a) or invaded (b) to the lower surface are shown. (c) Epithelial–mesenchymal transition-associated markers, E-cadherin and N-cadherin, were examined by Western blotting after cells were treated with or without CCL18 (20 ng/mL) for 2 or 3 days. β-Tubulin was used as an internal control. (d) Immunofluorescence staining of E-cadherin (red) and N-cadherin (green) was indicated in HSC6 and CAL33 cells with or without CCL18 for 3 days. Nuclear DNA was stained with DAPI (blue). Data are presented as the means ± SEM of at least three independent experiments, *P < 0.05 compared to the non-target control (NC).
overexpressed and positively correlated with advanced clinical stage in OSCC patients. Epithelial–mesenchymal transition was thought to play a key role in invasion and metastasis of many tumor types. To confirm whether CCL18 enhances OSCC invasion and metastasis through EMT, we selected two OSCC cell lines, HSC6 and CAL33, to undertake a Transwell assay. The results displayed that, after stimulation with 20 ng/mL CCL18, there were considerably more cells migrating or invading to the lower surface than the negative control (Fig. 1a,b). Moreover, we detected that the expression of the EMT-associated molecule, E-cadherin, an epithelial marker, decreased, whereas the expression of the mesenchymal marker, N-cadherin, increased in HSC6 and CAL33 cells after 3 days of treatment with CCL18 (Fig. 1c). In addition, the immunofluorescence staining of the cell lines showed the same results with Western blot analysis (Fig. 1d). Hence, our results indicated that CCL18 induced EMT and then promoted migration and invasion of OSCC cells.

Chemokine (CC motif) ligand 18 enhanced stem cell-like characteristics in OSCC. A growing number of studies have suggested that EMT is associated with the gain of molecular and functional traits of stem-like cells in normal and tumor cell populations. To confirm whether CCL18 enhances OSCC invasion and metastasis through EMT, we selected two OSCC cell lines, HSC6 and CAL33, to undertake a Transwell assay. The results displayed that, after stimulation with 20 ng/mL CCL18, there were considerably more cells migrating or invading to the lower surface than the negative control (Fig. 1a,b). Moreover, we detected that the expression of the EMT-associated molecule, E-cadherin, an epithelial marker, decreased, whereas the expression of the mesenchymal marker, N-cadherin, increased in HSC6 and CAL33 cells after 3 days of treatment with CCL18 (Fig. 1c). In addition, the immunofluorescence staining of the cell lines showed the same results with Western blot analysis (Fig. 1d). Hence, our results indicated that CCL18 induced EMT and then promoted migration and invasion of OSCC cells.

Chemokine (CC motif) ligand 18 enhanced stem cell-like characteristics in OSCC. A growing number of studies have suggested that EMT is associated with the gain of molecular and functional traits of stem-like cells in normal and tumor cell populations. Here, we detected the mRNA and protein expressions of stemness-related markers, OCT4 and Bmi-1, which
Slug (Snail2) are EMT-associated transcriptional factors. To identify whether the Snail and Slug were related to overexpressed CCL18, cells and CD133+ cells in HSC6 and CAL33 cells were significant to analyze the percentages of ALDHhigh+ cells and CD133+ were significantly upregulated in CCL18-treated cells compared to untreated cells (Fig. 2a). Flow cytometry was carried out to analyze the percentages of ALDHhigh+ cells and CD133+ cells, which are both used extensively to identify CSCs of oral cancer. The results showed that the percentage of ALDHhigh+ cells and CD133+ cells in HSC6 and CAL33 cells were significantly increased after treatment with CCL18 for 3 days (Fig. 2b). We also used a sphere formation assay to identify the stem-like cells and found that CAL33 cells, but not HSC6 cells, efficiently formed tumor spheroids (data not shown). The sphere-forming efficiency of CCL18-treated CAL33 cells was markedly promoted, and evaluated and quantified as average primary and secondary sphere numbers (Fig. 2c). SCC15 cells, another OSCC line, were also found to have enhanced sphere-forming capability in response to CCL18 (Fig. S1).

Table 1. Clinicopathological association of Bmi-1 expression in oral squamous cell carcinoma (OSCC) specimens

| Characteristics          | No. of cases | Bmi1 | P-value |
|--------------------------|--------------|------|---------|
| Age, years               |              |      |         |
| <50                      | 16           | 10   | 6       | 0.840  |
| ≥50                      | 29           | 19   | 10      |        |
| Gender                   |              |      |         |
| Male                     | 32           | 20   | 12      | 0.669  |
| Female                   | 13           | 9    | 4       |        |
| T – primary tumor        |              |      |         |
| T1 + T2                  | 26           | 14   | 12      | 0.082  |
| T3 + T4                  | 19           | 15   | 4       |        |
| N – regional lymph node  |              |      |         |
| Non-metastasis           | 38           | 17   | 11      | 0.502  |
| Metastasis               | 17           | 12   | 5       |        |
| Histological grade       |              |      |         |
| Well                     | 18           | 11   | 7       | 0.703  |
| Moderately + poorly      | 27           | 18   | 9       |        |
| Clinical stage           |              |      |         |
| I=II                     | 22           | 11   | 11      | 0.048  |
| III-IV                   | 23           | 18   | 5       |        |

Forty-five primary OSCC tissues were obtained from representative areas of each OSCC patient, postoperatively. Medical records for all OSCC cases were reviewed for clinical information. Bmi-1 expression was defined as positive when a typical nuclear staining was observed. The association between clinicopathology features and Bmi-1-positive expression was analyzed using the χ²-test. Bold text indicates significant.

It was then examined whether human OSCCs with high expression of CCL18 also showed features of stem-like cells. A total of 45 OSCC specimens were collected and used for immunohistochemical analysis. High positivity for CCL18 was associated with Bmi-1 positivity (P < 0.001; Fig. 2d). Representative immunostaining of consecutive tissue slides from two cases with CCL18 and Bmi-1 antibodies were shown in Figure 2(d). Taken together, these data provided evidence that CCL18 could contribute to enhance CSC features in OSCC.

Chemokine (CC motif) ligand 18 upregulated Slug expression by activating mTOR signaling. A proteomics analysis reported that CCL18-mediated invasion of ovarian cancer was strongly correlated with the mTORC2 pathway. Here, we explored the changes in mTOR signaling in HSC6 and CAL33 cells treated with CCL18. Western blot analyses showed that p-Akt (Thr308), p-Akt (Ser473), and p-mTOR were upregulated, whereas the total Akt and mTOR were unaffected (Fig. 4a). Next, INK128, a potent and selective mTOR ATP binding site competitor (Fig. S3), was used to block mTOR activation in cells before being treated with CCL18. We observed the mRNA and protein expressions of Slug were downregulated (Fig. 4b). These observations suggested that CCL18 activated mTOR signaling and then upregulated Slug expression in OSCC cells.

Inhibition of mTOR pathway reversed CCL18-induced EMT and stemness. To further investigate the role of the mTOR pathway in HSC6 and CAL33 cells with CCL18, we treated HSC6 and CAL33 cells with CCL18 in the presence of INK128, and detected that the expression of N-cadherin, OCT4, and Bmi-1 were decreased, but the expression of E-cadherin was increased, compared to that in the absence of INK128 (Fig. 5a). Immunofluorescence staining of E-cadherin and N-cadherin displayed the same results as Western blotting (Fig. 5b). Simultaneously, the proportions of ALDHhigh+ and CD133+ cells were downregulated (Fig. 5c), the migration and invasion ability (Fig. 5d,e) was weakened, and the forming spheres of CAL33 cells (Fig. 5f) and SCC15 cells (Fig. S1) were decreased, when the cells underwent CCL18 stimulation in the presence of INK128. All results showed that the mTOR inhibitor efficiently blocked CCL18-induced EMT and enrichment of stem-like cells.

Discussion

It is well established that chemokines in the tumor microenvironment play important roles in cancer progression. Our
previous study reported that chemokine ligand CCL18, predominantly produced by cancer epithelial cells, was abundantly expressed in primary OSCC tissues and associated with an advanced clinical stage. However, the mechanism of CCL18-stimulated oncogenesis and development in OSCC remained elusive. In this study, we showed that CCL18 accelerated the abilities of migration, invasion, and sphere formation, and upregulated expression of EMT and CSC-associated proteins.
markers in OSCC cell lines. Furthermore, we found a positive correlation between CCL18 and CSC biomarkers in human OSCC tissues. Moreover, we found the mTOR–Slug-dependent pathway was involved in these CCL18-induced activities, and silencing Slug or inhibiting mTOR signaling reversed CCL18-modulated EMT and stemness.

Recently, growing evidence has highlighted that CCL18 was overexpressed and related to tumorigenesis and metastasis in various cancers.\(^{23,24}\) Several groups have found that elevated CCL18 induced EMT in cancer cells.\(^{11,25–27}\) Here, our results showed that the high level of CCL18 accelerated the migration, invasion, and E- to N-cadherin switch of OSCC cells, which implied CCL18 was a vital factor involved in the induction of EMT in OSCC.

Accumulating evidence suggests that tumor cells that have undergone EMT display many similarities to CSCs, which are rare subtypes of cancer cells with unique abilities to self-renew and differentiate into progenitor cells, and are responsible for drug resistance, metastasis, and recurrence.\(^{15,16}\) Currently, OCT4, Bmi-1, ALDH, and CD133 are the common markers used to identify CSCs in OSCC.\(^{19,20}\) Our data showed that the mRNA and protein expressions of OCT4 and Bmi-1 were upregulated, as well as the percentages of ALDH\(^{+}\) cells and CD133\(^{+}\) cells after treatment with CCL18 in vitro. In addition,
Fig. 5. Inhibition of the mammalian target of rapamycin (mTOR) pathway reversed the chemokine (CC motif) ligand 18 (CCL18)-induced epithelial-mesenchymal transition and stemness in oral squamous cell carcinoma cells. (a–e) HSC6 and CAL33 cells were treated with CCL18 (20 ng/mL) and/or INK128 (100 nM) for 3 days. (A) Expression levels of E-cadherin, N-cadherin, OCT4, and Bmi-1 were detected by Western blot. α-Tubulin was used as an internal control. (b,c) Migration and invasion abilities were detected by Transwell assays. (d) Immunofluorescence staining of E-cadherin (red) and N-cadherin (green) is indicated. Nuclear DNA was stained with DAPI (blue). (e) Percentages of aldehyde dehydrogenase (ALDH)high cells and CD133+ cells were detected by flow cytometry. (f) CAL33 cells were cultured in low-adherence conditions to form spheres in 6-well plates exposed in CCL18 (20 ng/mL) in the absence or presence of INK128. Average numbers of primary and secondary spheres (>100 μm) were calculated for each well (right). All the data are presented as the means ± SEM of at least three independent experiments. *P < 0.05 compared to NC; **P < 0.05 compared to CCL18.
CSCs are characterized by their tumorsphere-forming ability in vitro, and tumorsphere formation assays are widely used to identify, isolate, enrich, maintain, or expand potential CSC subpopulations from various types of cancers.\(^{(26)}\) Interestingly, we found that CAL33 and SCC15 cells, but not HSC6 cells, efficiently formed tumorspheres, which was also strengthened by the high level of CCL18. The reasons might be the heterogeneity of normal stem cells where these CSCs originated, the presence of several different activated signaling pathways, or the different expression patterns of various CSC markers in each OSCC subtype. Other groups also discovered that only a few cancer cell lines could establish stable spheres, such as one-third of thyroid cancer cell lines\(^{(29)}\) and one-quarter of hepatocellular carcinoma cell lines\(^{(30)}\). Furthermore, we found high levels of CCL18 and Bmi-1 positivity were significantly associated in human OSCC specimens. These results provided evidence that CCL18 stimulation enhanced the stem cell-like characteristics of OSCC cells.

Slug (Snail2) is a member of the Snail family of zinc-finger transcription factors, which could bind specifically to a subset of E-box motifs in target promoters, such as the E-cadherin promoter, and are key mediators of EMT and CSC enrichment in many tumors.\(^{(31,32)}\) In this study, we found that CCL18 upregulated Slug mRNA and protein expressions, and Slug knockdown by RNAi blocked EMT and stemness of HSC6 and CAL33 cells, which proved that Slug was required to induce theses phenotypes in OSCC cells.

Mammalian target of rapamycin is a Ser/Thr kinase, and its activity is misaligned in several human diseases, including cancer.\(^{(33)}\) There is no doubt that the classical pathway of PI3K/Akt/mTOR signaling, one of the most important intracellular pathways, is frequently activated in diverse cancer types. Disorders of the PI3K/Akt/mTOR signaling pathway mediated through molecular aberrations contribute to tumor development and therapeutic resistance, as well as CSC biology.\(^{(34,35)}\) In this study, we detected that elevated CCL18 upregulated Slug expression to modulate EMT and the gain of stem cell-like properties by promoting the phosphorylation of Akt and mTOR, and these effects could be blocked by INK128 (a potent and selective TORC1/2 dual inhibitor) in vitro. Hence, these results highlighted that the CCL18/mTOR/Slug pathway is involved in the regulation of EMT and stemness in OSCC.

The underlying mechanisms of cancer often contain a series of complex aberrations that stimulate critical cellular signaling pathways in oncogenesis. Therefore, CCL18-related signaling may also relate to more than Akt/mTOR/Slug pathway in cancer. Zhang et al.\(^{(36)}\) reported that CCL18 binding to Nir1 activated two signaling pathway in breast cancer: (i) the Akt/LIMK/cofilin pathway, which regulates actin polymerization and rearrangement of the cell cytoskeleton; and (ii) the Akt/GSK3β/Snail pathway, which induces EMT. Lin et al.\(^{(37)}\) suggested that CCL18-induced endothelial-mesenchymal transformation and pro-angiogenesis through ERK and Akt/GSK3β/Snail signaling in breast cancer. These reports and our study commonly implied that Akt may be a key node in CCL18 facilitating cancer development. Interestingly, in our study, the CCL18-related transcription factor that induced EMT in OSCC was Slug, not Snail, as in breast cancer, which reflected the heterogeneity between different tumors. In addition, ERK1/2/NF-κB, Pyk2/Src, or ELMO1/ Dock180 signaling were stimulated by CCL18 and then regulated migration and invasion in some carcinomas.\(^{(25–28,38–40)}\) Above all, CCL18 might be involved in multiple intracellular signaling participating in cancer development, and further research is required to explore the function and mechanisms of CCL18 in different cancer types.

In conclusion, increased CCL18 activated mTOR signaling to upregulate Slug expression level, and subsequently modulated EMT and stem cell-like characteristics in OSCC. Thus, our findings not only provide new insight into the role of CCL18 in the development of cancer but also offer new potential therapeutic targets for early diagnosis and treatment of OSCCs.

Acknowledgment

This work was supported by grants from the National Natural Science Foundation of China (Nos. 81371148 and 81671000).

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| Akt          | protein kinase B |
| ALDH         | aldehyde dehydrogenase |
| CCL18        | chemokine (CC motif) ligand 18 |
| CSC          | cancer stem(-like) cell |
| EMT          | epithelial-mesenchymal transition |
| GSK3β        | glycogen synthase kinase 3β |
| mTOR         | mammalian target of rapamycin |
| OCT4         | octamer-binding transcription factor 4 |
| OSCC         | oral squamous cell carcinoma |
| PI3K         | phosphatidylinositol 3-kinase |
| qRT-PCR       | reverse transcription-quantitative real-time PCR |
| Si-NC        | non-target control siRNA |

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