

CCL18-NIR1/GPR3 promotes oral cancer cell growth and metastasis by activating the JAK2/STAT3 signalling pathway

Guodong Chen (✉ chenzejia99@163.com)

Shunde Hospital, Southern Medical University (The First People's Hospital of Shunde)

<https://orcid.org/0000-0003-2331-8022>

Xiao Jiang

Stomatological Hospital, Southern Medical University

Xiang Sun

Stomatological Hospital, Southern Medicine University

Zhijie Huang

Stomatological Hospital, Southern Medical University

Xianghuai Zheng

Stomatological Hospital, Southern Medical University

Jingpeng Liu

Stomatological Hospital, Southern Medical University

Jun Shen

Stomatological Hospital, Southern Medical University

Bo Jia

Stomatological Hospital, Southern Medical University

Haiyun Luo

Stomatological Hospital, Southern Medical University

Zhaoyi Mai

Stomatological Hospital, Southern Medical University

Jianjiang Zhao

Stomatological Hospital, Southern Medical University

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Abstract

Background Chemokine (C-C motif) ligand 18 (CCL18) affects the malignant progression of varying cancers by activating chemokine receptors. Our previous study have shown that CCL18 promotes hyperplasia and invasiveness of oral cancer cells; however, the cognate receptors of CCL18 involved in the pathogenesis of oral squamous cell carcinoma (OSCC) has not yet been identified. This study aimed to investigate the underlying molecular mechanisms through which CCL18 promotes OSCC progression by binding to specific functional receptors. **Methods** The properties of CCL18 receptors (i.e., NIR1, CCR6, CCR8, and GPR3) in OSCC were detected by conducting western blotting, immunofluorescence, and immunocytochemistry assays. The binding between CCL18 and its receptors was verified by performing coimmunoprecipitation (CO-IP) assays. The χ^2 test was applied to analyze the relationship between CCL18 receptor expression patterns and clinicopathological factors. Recombinant CCL18 (rCCL18) and receptor siRNA were used to confirm the effects of CCL18 receptor axis on the morphology of cancer cells (i.e., proliferation, and metastasis), epithelial-mesenchymal transition (EMT) and the expression of the JAK2/STAT3 signalling pathway. **Results** NIR1 and GPR3 as specific receptors of CCL18 in OSCC were found to be significantly increased and positively related to the TNM stage of OSCC patients. rCCL18 induced phenotype alterations of oral cancer cells including cell growth, metastasis, and EMT. Knockdown of NIR1 and/or GPR3 expression could block the effects of rCCL18-induced OSCC. Furthermore, JAK2/STAT3 signalling was confirmed to be a downstream pathway of the CCL18-NIR1/GPR3 axis. **Conclusion** CCL18 can promote the progression of OSCC by binding specific receptors (NIR1 and GPR3), and the CCL18-receptor signalling can activate JAK2/STAT3 pathway. The identification of the mechanisms of CCL18 promoting OSCC progression could implicate potential therapeutic targets for treating oral cancer.

Background

Oral squamous cell carcinoma (OSCC) as the most common type of oral cancer is well-known to be characterized by a high risk of local invasion and cervical lymph node metastasis [1]. Chemokines have been shown to promote the metastasis of OSCC by facilitating the proliferation, survival, and migration of cancer cells, as well as shaping the tumour microenvironment [2]. Chemokine receptors can selectively bind to chemokines and further transduce intercellular signals that can impact the oncogenic processes of cancer [3]. Chemokines-receptors signalling axis has been therefore considered as the hallmark of cancer and potential therapeutic strategy for cancer treatment [4]. CCL18 (Chemokine (C-C motif) ligand 18) as one of the most commonly investigated homeostatic chemokine have been shown to promote the progression of various cancers (e.g., lung cancer [5], bladder cancer [6], pancreatic ductal adenocarcinoma [7], and breast cancer [8]) by implicating in the activation of CCL18-receptors signalling axis. The experimentally validated receptors of CCL18 includes NIR1 (N-terminal domain interacting receptor 1) [8], CCR8 (C-C chemokine receptor type 8) [6], CCR6 (C-C chemokine receptor type 6) [5], and GPR3 (G-protein coupled receptor 3) [7]. A previous research [9] conducted by our team has shown the involvement of CCL18 in the metastasis of OSCC by promoting the growth and invasion of cancer cells;

however, researchers are still lack of understanding about the involvement of CCL18-receptor signalling axis in the cancer biology of OSCC. It remains a question that if the dysregulation of the receptors listed above (e.g., NIR1, CCR6, CCR8 and GPR3) are also involved in the progression of OSCC by binding to CCL18. Consequently, the identification of specific receptors of CCL18 appears to be essential for researchers to obtain a better understanding of the implication of CCL18-receptors signalling pathway in OSCC.

The signalling pathways are well-known to interact with each other by activating or inhibiting their downstream pathways, thereby forming a complicated pathway interaction network to influence the immune regulation of cancer [10]. Based on this theory, we believe that it is necessary to identify the downstream pathway of CCL18-receptors signalling pathway. JAK2/STAT3 (Janus kinase 2/signal transducers and activators of transcription 3) signalling as an oncogenic pathway involved in many solid cancers including OSCC [11] has been shown to be activated by several chemokine-signalling axes, for instance, CXCL12-CXCR4 axis [12], CXCL8-CXCR1/CXCR2 axis [13], and CXCL9-CXCR3 axis [14]. The interaction between JAK2/STAT3 and these chemokine-receptor axes arouse our interest and made us wonder: if the CCL18-receptors signalling is coupled with the JAK2/STAT3 pathway and if the interaction between both pathways plays a contributing role in the metastasis of OSCC. To our knowledge, this is the first report that identified the specific receptors of CCL18 that are involved in the regulation of OSCC, and further investigated the underlying mechanism of CCL18-receptor axis in promoting metastasis of OSCC by determining its coupling downstream pathway.

Methods

Patients and samples

Twenty-five patients with OSCC underwent surgical resection at the Department of Craniofacial Surgical Resection, Stomatological Hospital, Southern Medical University. Primary OSCC tissues (n=25) and some adjacent normal tissues (n=10) were obtained postoperatively. All patients provided informed consent. The study was approved by the Ethics Committee of Stomatological Hospital, Southern Medical University. Another 18 OSCC tissue samples were acquired from tissue chips with detailed clinical information that were purchased from WoZhe Biotechnology Company Ltd. (Guangzhou, China).

Cell lines and reagents

The HSC6 cell line was purchased from CinoAsia Co., Ltd. (Shanghai, China). CAL27, SCC9 and HOK cell lines were purchased from TongPai Biotechnology Co., Ltd. (Shanghai, China). OSCC cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin, and HOK cells were cultured in KSFM (Gibco, USA). Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Recombinant human CCL18 (rCCL18) was purchased from Peprotech (Peprotech, Inc., Princeton, NJ, USA).

Immunohistochemistry

The OSCC tissues and adjacent normal tissues were analysed using immunohistochemistry (IHC). Tissues were dewaxed in xylene and rehydrated in a graded alcohol series. After antigen retrieval with Tris-EDTA, the slides were blocked with 5% serum. Primary antibodies against NIR1 (1:100, Novus, Littleton, CO, USA), GPR3 (1:100, Abcam, Cambridge, MA, UK), CCR6 (1:100, Invitrogen, Carlsbad, CA, USA) and CCR8 (1:100, Abcam, UK) were incubated overnight at 4°C. The sections were covered with secondary antibody and incubated at room temperature for 30 min. Next, the tissue sections were visualized with DAB (Gene, Shanghai, China). The expression of NIR1 and GPR3 was quantified using a visual grading system based on the average optical density (+: positive, ++: strong positive).

Immunofluorescence

Cells were seeded in glass bottom cell culture dishes for 24 h. Then, the cells were rinsed with PBS and fixed with 4% paraformaldehyde solution for 30 min, permeabilized with 0.3% Triton X-100 for 15 min, and blocked with 5% bovine serum albumin (BSA) for 1 h. Subsequently, the cells were incubated overnight at 4°C with the following primary antibodies: NIR1 (1:200, Novus, USA), GPR3 (1:50, Abcam, UK), CCR6 (1:200, Invitrogen, USA), and CCR8 (1:100, Abcam, UK). The next day, the samples were incubated with secondary antibody (1:500, Abcam, UK) in the dark for 1 h and counterstained with DAPI (Invitrogen, USA) for 5 min. The results were photographed with an automated upright microscope system (Leica, DM4000B Leica Microsystems, Wetzlar, Germany).

Transfection of NIR1/GPR3 siRNA

For transfection, HSC6 cells and CAL27 cells were seeded in 6-well plates at 2×10^5 /well. siRNA against NIR1 (siNIR1) or GPR3 (siGPR3) was transferred into cells with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. A negative siRNA (siNC) sequence was used as a control. Silencing efficiency was verified by qRT-PCR and Western blotting after 48 h of transfection. The following three interfering sequences for NIR1 and GPR3 were synthesized by GenePharma (Jiangsu, China):

siNIR1-1: 5'-CACGCCCAAAGAAGAACAATT-3'

siNIR1-2: 5'-GUGGUCGCAUCACAUACAATT-3'

siNIR1-3: 5'-CCAUCUGCUCUGAGGCCUUUTT-3'

siGPR3-1: 5'-GCUACCUUUCUCUGUACAATT-3'

siGPR3-2: 5'-GCAUCAUGCUGCAGCUCUATT-3'

siGPR3-3: 5'-GCAACCAGGAUGUGCAGAATT-3'

Western blot analysis

Cells and tissues were lysed in cell lysis buffer with phosphatase inhibitor, protease inhibitor and PMSF (KeyGEN BioTECH, Jiangsu, China). Protein levels were measured using a BCA protein assay kit (Cwbiotech, Jiangsu, China). Twenty micrograms of protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane (Merck KGaA, Darmstadt, Germany). The PVDF membrane was blocked with 5% BSA (Pierce, Rockford, IL, USA) for 1 h and then incubated with the following primary antibodies at 4°C overnight: NIR1 (1:2000, Novus, USA), GPR3 (1:1000, Abcam, UK), CCR6 (1:250, Novus, USA), CCR8 (1:2000, Abcam, UK), GAPDH (1:1000, Abcam, UK), E-cadherin (1:1000, CST, Danvers, MA, USA), N-cadherin (1:1000, CST, USA), ZEB2 (1:1000, Merck KGaA, Germany), JAK2 (1:1000, CST, USA), P-JAK2 (Tyr1007/1008) (1:1000, CST, USA), STAT3 (1:1000, CST, USA), P-STAT3 (Tyr705) (1:1000, CST, USA), and β -actin (1:1000, Abcam, UK). Then, the PVDF membrane was incubated with secondary antibody (1:2000, Abcam, UK). The expression of the protein bands was detected by ultrasensitive chemiluminescence imaging, and Image Lab software was used to analyse the density of each band.

qRT-PCR

Cells were collected, and total RNA was extracted using TRIzol reagent (Invitrogen, USA). Complementary DNA (cDNA) was synthesized using FastKing gDNA Dispelling RT SuperMix (TIANGEN, Beijing, China). qPCR was performed with Talent qPCR PreMix (TIANGEN, China) and a CFX96™ Connect Real-Time System (C1000 Touch™ Thermal Cycler, BIO-RAD, Hercules, CA, USA). The thermocycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 15 sec at 60°C. The relative levels of mRNA expression were normalized to GAPDH expression using the $2^{-\Delta\Delta Cq}$ method. The primers were as follows: NIR1: (Forward: GATGCCAGAGGAGAAGGGAC; Reverse: TCGCTGTCTTCGTGGATCTC), GPR3: (Forward: CTCCACGGTTCCAGAATGTT; Reverse: GGGAGAAGGCTCTGGTTTCT), GAPDH: (Forward: CTCCTCCTGTTTCGACAGTCAGC; Reverse: CCCAATACGACCAAATCCGTT).

Coimmunoprecipitation assay

Protein coimmunoprecipitation (CO-IP) assays were performed using a Pierce™ Crosslink Magnetic IP/CO-IP Kit (Thermo Fisher Scientific, Waltham, MA, USA). Cells were lysed in IP lysis/wash buffer for 5

min and then centrifuged at 4°C for 15 min. Magnetic beads were coated with anti-CCL18 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-GPR3 (Santa Cruz Biotechnology Inc., USA) or anti-IgG (CST, USA). After crosslinking the antibodies with DSS, the cancer cell lysates and prepared magnetic beads were mixed at 4°C overnight. After washing with IP lysis/wash buffer and ultrapure water, the CCL18-associated and GPR3-associated proteins were eluted with elution buffer and detected by Western blotting.

CCK-8 assay

Cells were treated with siRNA-NIR1 and/or siRNA-GPR3 for 48 h, and 5000 cells were then added to 96-well plates and treated with 20 ng/ml rCCL18. At 24 h, 48 h and 72 h, CCK-8 reagent (Sigma-Aldrich, Louis, MO, USA) was added, and the absorbance values of each well were read with a microplate reader (Thermo Fisher Scientific. Waltham, USA) at 450 nm.

Clone formation assay

Forty-eight hours after siRNA-NIR1 and/or siRNA-GPR3 transfection, cells were plated in 6-well plates at 1000 cells per well and exogenously stimulated with 20 ng/ml rCCL18. The number of cell clones was counted by crystal violet staining 14 days later.

Transwell assays

Cell migration and invasion were detected by transwell assays (Corning, New York, NY, USA). The upper chamber was precoated with 50 µl 20% Matrigel (Gibco, USA) for the invasion assay. Cells were transfected with siRNA for 48 h and then suspended in serum-free medium with or without 20 ng/ml rCCL18. The prepared cells were seeded in the upper insert, and the lower chamber was filled with DMEM containing 15% FBS. Then, the transwell plates were incubated at 37°C with 5% CO₂ for 24 h. Cells that did not invade through the pores were gently removed with cotton tips. The upper chamber was fixed with 4% formaldehyde for 15 min and stained with a 0.4% crystal violet solution for 15 min. Five randomly selected fields of view at ×50 magnification were photographed under a light microscope (Carl Zeiss AG, Oberkochen, Germany).

Statistical analysis

Data statistical analysis was performed using GraphPad Prism 7.00 software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 20 (IBM Corporation, Armonk, NY, USA). The data are presented as

the means \pm SEM based on three replicates per group. χ^2 testing was used to analyse the relationship between NIR1/GPR3 and the clinical information of OSCC patients. Student's t test and one-way ANOVA were used to detect the mean difference of different samples. $P<0.05$ was considered statistically significant.

Results

NIR1 and GPR3 expression in OSCC and its clinical significance

Differential expression levels of NIR1, GPR3, CCR6 and CCR8, which are receptor-binding proteins of CCL18, have been reported in different tumours. We used immunohistochemistry (IHC) to detect NIR1, GPR3, CCR6 and CCR8 expression in 10 pairs of OSCC tissues and adjacent normal tissues. Positive NIR1 and GPR3 expression was found to be localized primarily in the cellular membrane and cytoplasm of oral cancer cells. CCR6 and CCR8 expression was not found in oral cancer cells or normal oral mucosa cells (Fig. 1a). Western blotting results showed that the expression of NIR1 and GPR3 was significantly higher in cancer tissues than in adjacent normal tissues. CCR6 and CCR8 protein expression in cancer tissues and adjacent tissues was low (Fig. 1b). To further evaluate the clinical significance of NIR1 and GPR3, 43 OSCC tissues were analysed with NIR1 and GPR3 antibodies for IHC. All OSCC tissues displayed positive NIR1 and GPR3 expression. Highly NIR1- or GPR3-positive tissues were closely related to the clinical TNM stage ($P=0.042$, Table 1; $P=0.040$, Table 2; Fig. 2a and b). However, the expression of NIR1 and GPR3 was not significantly related to other clinical features of the patients, such as age, sex or histological grade.

In addition, we examined the protein levels of NIR1, GPR3, CCR6 and CCR8 in 3 OSCC cell lines (HSC6, CAL27 and SCC9) and in normal human oral epithelial keratinocytes (HOK). Western blotting and immunofluorescence (IF) verified that NIR1 and GPR3 were highly expressed in all OSCC cells compared with HOK cells and that CCR6 and CCR8 were rarely expressed in OSCC cells and HOK cells (Fig. 3a and c). qRT-PCR also showed increased mRNA expression of NIR1 and GPR3 in OSCC cells (Fig. 3b).

NIR1 is the most common receptor of CCL18, and their potent combination has been verified in breast cancer [15]. GPR3, as a receptor of CCL18, has been implicated in pancreatic cancer. However, there has been no experimental evidence that CCL18 and GPR3 bind effectively. We immunoprecipitated total protein extracts from CAL27 cells with anti-CCL18 or anti-GPR3 antibodies. As shown in Fig. 3d, Western blotting revealed specific bands for CCL18 or GPR3 in the precipitated proteins. Neither of these two proteins appeared when an irrelevant antibody (IgG) was used for immunoprecipitation. These data suggest that NIR1 and GPR3 may be receptors of CCL18 and thus participate in the progression of OSCC.

Effective interference sequences of NIR1 and GPR3 in OSCC cells

We next sought to confirm that CCL18 regulates the progression of OSCC through NIR1 and GPR3. Three different siNIR1 segments and siGPR3 segments were designed to screen effective interference

sequences. Our results showed that the mRNA levels of NIR1 and GPR3 were significantly decreased by siNIR1-1, siNIR1-2, and siNIR1-3 and siGPR3-2 (Fig. 4a). Western blotting results showed that siNIR1-1 and siNIR1-3 could decrease the protein level of NIR1 and that siGPR3-1, siGPR3-2, and siGPR3-3 could decrease the protein expression of GPR3 (Fig. 4b). However, there were no significant changes in mRNA or protein levels in untreated cells or cells transfected with negative siRNA sequences. Overall, the data suggested that siNIR1-3 and siGPR3-2 were effective sequences for subsequent experiments.

NIR1 and GPR3 are required for OSCC cell proliferation via CCL18

To investigate the function of the CCL18-NIR1/GPR3 axis in OSCC, HSC6 and CAL27 cells were transfected with siNIR1 and/or siGPR3 and then stimulated with 20 ng/ml rCCL18. The proliferation of each group of cells was detected by CCK8 and clone formation assays. CCK8 analysis results showed that the proliferation of OSCC cells increased upon rCCL18 stimulation for 48 h and 72 h. After knocking down NIR1 and/or GPR3, the proliferation effect of rCCL18 on OSCC cells was reduced (Fig. 5a). As shown in Fig. 5b, OSCC cells cultured with rCCL18 had strong clone formation ability for 14 days. Compared to the control conditions, transfection with siNIR1 and/or siGPR3 in cells cultured with rCCL18 resulted in a significant decrease in colony numbers.

NIR1 and GPR3 are required for cell mobility and EMT in OSCC cells via CCL18

NIR1 and GPR3 were associated with the tumour stage in OSCC patients. We thus characterized the effects of the CCL18-NIR1/GPR3 axis on the migration and invasion of OSCC cells using a transwell assay. HSC6 and CAL27 cells were treated with rCCL18, siNIR1+rCCL18 and/or siGPR3+rCCL18; untreated cells and siNC+rCCL18 treatment served as controls. The results in Fig. 6a show that rCCL18 could promote OSCC cell migration through the transwell membrane. In the presence of siNIR1 and/or siGPR3, the number of cells on the submembrane surface decreased despite rCCL18 stimulation. Similar results were observed in the cell invasion assay (Fig. 6b).

EMT plays a critical role in oral cancer metastasis by enhancing migration and invasion. Therefore, we examined the expression of E-cadherin, N-cadherin and ZEB2 in HSC6 and CAL27 cells subjected to the different treatments stated above. Compared with that in the untreated group, the expression of E-cadherin decreased, and the expression of N-cadherin and ZEB2 increased in the rCCL18 group. Knocking down NIR1 and/or GPR3 reversed the decreases in E-cadherin levels and the increases in N-cadherin and ZEB2 levels caused by rCCL18 (Fig. 6c). All data suggested that CCL18 enhanced the EMT property of OSCC by binding to the receptors NIR1 and GPR3 and then promoting OSCC cell invasion and migration.

CCL18-NIR1/GPR3 activates the JAK2/STAT3 signalling pathway in OSCC cells

The JAK2/STAT3 pathway is known to be involved in the growth and EMT process in OSCC. As shown in Fig. 7, P-JAK2 (Tyr1007/1008) and P-STAT3 (Tyr705) levels increased, but there was no change in JAK2 and STAT3 protein expression in rCCL18-treated OSCC cells. Silencing NIR1 and/or GPR3 in HSC6 and CAL27 cells could reverse the activation effect of rCCL18 on JAK2 and STAT3. In addition, decreased STAT3 expression was found in HSC6 and CAL27 cells treated with siNIR1+siGPR3+rCCL18. These findings indicated that CCL18-NIR1/GPR3 could promote the malignant progression of OSCC by activating the JAK/STAT3 signalling pathway.

Discussion

Our previous studies showed that CCL18 which was predominantly secreted by oral cancer cells promoted the malignancy of OSCC. A unique feature of chemokines has been commonly demonstrated to be the nature of receptors to which they bind. Chemokines have been shown to elicit their effects mainly by activating specific transmembrane receptors which belong to the large family of G protein-coupled receptors (GPCRs) [16]. The receptor of CCL18 involved in mediating tumour pathogenesis has been investigated in many cancers; however, the specific receptor of CCL18 in oral cancer remains uncertain. In this study, we examined the expression profile of several putative receptors of CCL18 (i.e., NIR1, GPR3, CCR6, and CCR8) in OSCC tissues and cells through multiple immunoassays. Our results revealed that NIR1 and GPR3 were significantly upregulated in OSCC samples, whereas CCR6 and CCR8 were downregulated in both OSCC and control specimens. Additionally, clinical analysis of 43 OSCC patients showed that the expression of NIR1 and GPR3 was higher in TNM stage III/IV samples compared to TNM stage I/II samples, suggesting close association between NIR1 and GPR3 and malignancy of oral cancer.

This section will discuss the potential roles of NIR1 and GPR3 respectively in oral cancers by interacting with CCL18. Taking the example of NIR1, this receptor as a member of putative six transmembrane GPCRs has been firstly confirmed to be dysregulated in breast cancer [15]. Apart from its aberrant expression in breast cancer, the overexpression of NIR1 was also found to be associated with the clinical stage and histological grade of hepatocellular carcinoma (HCC) [17]. In addition, CCL18-NIR1 axis has been suggested to activate the intracellular calcium signaling and further promote the growth and invasion of breast and lung cancer cells [8, 18]. Looking at the case of GPR3, this receptor belongs to the family of constitutively active Gs-coupled receptors and is predominantly expressed in both brain and oocytes [19]. Previous investigations about GPR3 focused mainly on its involvement in nervous system and follicle development in vertebrates [20]. Most studies showed that GPR3 belongs to orphan receptor and thus is lack of endogenous ligand [21]. Surprisingly, however, another study implied that abnormal expression of GPR3 acts as a receptor of CCL18 in pancreatic ductal adenocarcinoma (PDAC) [7]. This study for the first time reports the crosslink between CCL18 and GPR3 in OSCC. Our findings unveiled critical potential of interaction between CCL18 and GPR3 in the chemokine-receptor network.

To further explore the role of CCL18-NIR1/GPR3 in oral cancer, the OSCC models of NIR1 and/or GPR3 silencing were established. It was observed that depletion of NIR1 and/or GPR3 in HSC6 and CAL27 cells

impaired CCL18-induced proliferation, migration and invasion. Furthermore, expression of EMT markers was also altered. While rCCL18 treatment led to down-regulation of E-cadherin and elevation of N-cadherin and ZEB2 in OSCC cells, silencing NIR1 and/or GPR3 caused the opposite, indicating that NIR1 and GPR3 may play essential roles in the CCL18-induced malignant progression of OSCC.

JAK2/STAT3 signalling is an evolutionarily conserved signalling pathway and known to interfere with tumour growth and metastasis of OSCC [11, 22]. Activation of JAK2 protein kinase can catalyze STAT3 protein phosphorylation which regulates the expression of oncogenic genes [23]. E-cadherin, N-cadherin and ZEB2 are well-known downstream molecules of JAK2/STAT3 signalling pathway [24-26]. Numerous studies have authenticated that JAK2/STAT3 can be activated by certain chemokines, including CXCL3, IL-6, CXCL9 and CCL20 [27-30]. However, the relationship between CCL18 and JAK2/STAT3 signalling pathway remains elusive. In our study, we found that both P-JAK2 and P-STAT3 were upregulated in rCCL18-stimulated oral cancer cells. But such CCL18-induced JAK2/STAT3 activation can be considerably diminished by siNIR1 and/or siGPR3 treatment. Intriguingly, when NIR1 and GPR3 were silenced simultaneously, the total protein levels of STAT3 in HSC6 and CAL27 cells were also significantly decreased. Overall, abovementioned results indicated that JAK2/STAT3 signalling contributes to the CCL18-NIR1/GPR3 mediated proliferation and migration in OSCC.

It is worthwhile to clearly state the limitation of this study. First, the relationship between NIR1 and GPR3 was not investigated in this study, in other words, if they play synergistically or antagonistically in OSCC needs to be elucidated in the future study. Another limitation that needs to be acknowledged is the 5-year survival rate of OSCC patients was not reported in this study since several patients fail to participate in the follow-up appointments. However, this study also provides implications for future research. First, the biomarkers identified in this study could be used as therapeutic targets in the gene delivery therapy of OSCC. In addition, the identification of these critical biomarkers will also advance the development of multi-target drugs; however, if these target drugs could synergistically work with conventional chemotherapeutic agents remains a question. Furthermore, these biomarkers related to metastasis could be regarded as novel evaluation indexes for prognosis of OSCC. The testing kits based on these biomarkers could be also developed for guiding clinical practitioners to promptly make subsequent treatment plans individually for patients with poor prognosis.

Conclusion

In summary, two receptors (i.e., NIR1 and GPR3) of CCL18 were identified to be upregulated in OSCC and associated with advanced tumour stage of OSCC. The CCL18-NIR1/GPR3 axis was found to regulate the proliferation, metastasis and EMT of OSCC cells by activating JAK2/STAT3 signalling pathway. Our results could facilitate a better understanding of the underlying mechanisms of CCL18-induced OSCC progression, and also indicate that NIR1 and GPR3 might be potential therapeutic targets for treating oral cancer.

Abbreviations

OSCC: Oral squamous cell carcinoma; CCL18: Chemokine (C-C motif) ligand 18; NIR1: N-terminal domain interacting receptor 1; CCR8: C-C chemokine receptor type 8; CCR6: C-C chemokine receptor type 6; GPR3: G-protein coupled receptor 3; rCCL18: Recombinant CCL18; JAK2/STAT3: Janus kinase 2/signal transducers and activators of transcription 3; P-JAK2: Phosphorylation of JAK2; P-STAT3: Phosphorylation of STAT3; EMT: Epithelial-mesenchymal transition; CO-IP: Coimmunoprecipitation.

Declarations

Author contributions

JX designed and performed the experiments, and prepared the manuscript. LJP, LHY, SJ and JB participated in study design and. SX, ZXH, HZJ and MZY performed the experiments and data analysis. ZJJ and CGD approved the manuscript editing. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

The project was approved by the Ethics Committee of Stomatological Hospital, Southern Medical University. All patients provided written informed consent in accordance to the Stomatological Hospital, Southern Medical University Ethics Committee protocols.

Consent for publication

Not applicable

Conflict of interest

None declared.

Author details

¹Department of Oral Medicine, Stomatological Hospital, Southern Medical University, Guangzhou, Guangdong, China. ²Shunde Hospital, Southern Medical University (The First People's Hospital of Shunde), Foshan, Guangdong, China.

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Tables

Due to technical limitations the tables are available as a download in the Supplementary Materials.

Figures



Figure 1

High expression of NIR1 and GPR3 in OSCC cancer. A Representative images of NIR1, GPR3, CCR6 and CCR8 staining in OSCC tissues and adjacent normal tissues (magnification 100×). B Western blotting analysed that the protein levels of NIR1 and GPR3 were up-regulated in oral cancer tissues (**P<0.01, ***P<0.001 vs. adjacent normal tissues). CCR6 and CCR8 were negative expressed in OSCC tissues and control. The data represent mean ± SEM of three independent experiments.



Figure 2

Positive NIR1 and GPR3 expression in OSCC tissues is associated with cancer cells metastasis. 43 OSCC cases were stained with NIR1 antibody or GPR3 antibody, respectively. Representative IHC images showed that NIR1 and GPR3 were more expressed in metastatic cases of OSCC than in non-metastatic cases (magnification 50×).



Figure 3

As potential receptors of CCL18, NIR1 and GPR3 are highly expressed in OSCC cell lines. A Western blotting showed that NIR1 and GPR3 were increased expressed in OSCC cell lines (HSC6, CAL27, SCC9), compared with HOK cells. CCR6 and CCR8 were rarely expressed in OSCC cell lines and HOK cells (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. HOK). B qRT-PCR detected the mRNA levels of NIR1 and GPR3 were elevated in OSCC cells lines. C Immunofluorescence staining of NIR1, GPR3, CCR6 and CCR8

(red) and nuclei (blue) in oral cancer cells (HSC-6, CAL27 and SCC9) and HOK cells (magnification 200×). D CO-IP verified the effective binding between CCL18 and GPR3.



Figure 4

Effective siRNA segments of NIR1 and GPR3 were screened in HSC6 cells. Western blotting and qRT-PCR measured the NIR1 or GPR3 protein expression A and mRNA expression B in HSC6 cells, which were transfected with siNIR1-1, siNIR1-2, siNIR1-3, siGPR3-1, siGPR3-2 and siGPR3-3, respectively. (*P<0.05 vs. siNC control)



Figure 5

CCL18-NIR1/GPR3 affects the proliferation of OSCC cells. Cells were treated with rCCL18, siNC+ rCCL18, siNIR1+ rCCL18, siGPR3+ rCCL18, siNIR1+ siGPR3+ rCCL18. The untreated cells and siNC+rCCL18 cells as control. A CCK8 assay analysed the proliferation of cells which in different treatment groups at 24 h, 48 h and 72 h. B HSC6 cells and CAL27 cells were treated as above. Continuous stimulation of 20 ng/ml rCCL18 for 14 days and cells were stained with 0.4% crystal violet. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. control)



Figure 6

CCL18-NIR1/GPR3 promotes the locomotion and EMT of OSCC cells. HSC6 cells and CAL27 cells were treated with rCCL18, siNC+rCCL18, siNIR1+rCCL18, siGPR3+rCCL18, siNIR1+siGPR3+rCCL18. The untreated cells and siNC+rCCL18 cells as control. A and B The effects of CCL18-NIR1/GPR3 axis on the migration and invasion of OSCC cells. Oral cancer cells at the bottom of the transwell membrane were stained with 0.4% crystal violet. Five fields per hole were randomly selected under the microscope at 50×. C The protein expression of E-cadherin, N-cadherin and ZEB2 in HSC6 and CAL27 cells which subjected to the different treatment stated above were tested by western blotting, β -actin was used as internal control.

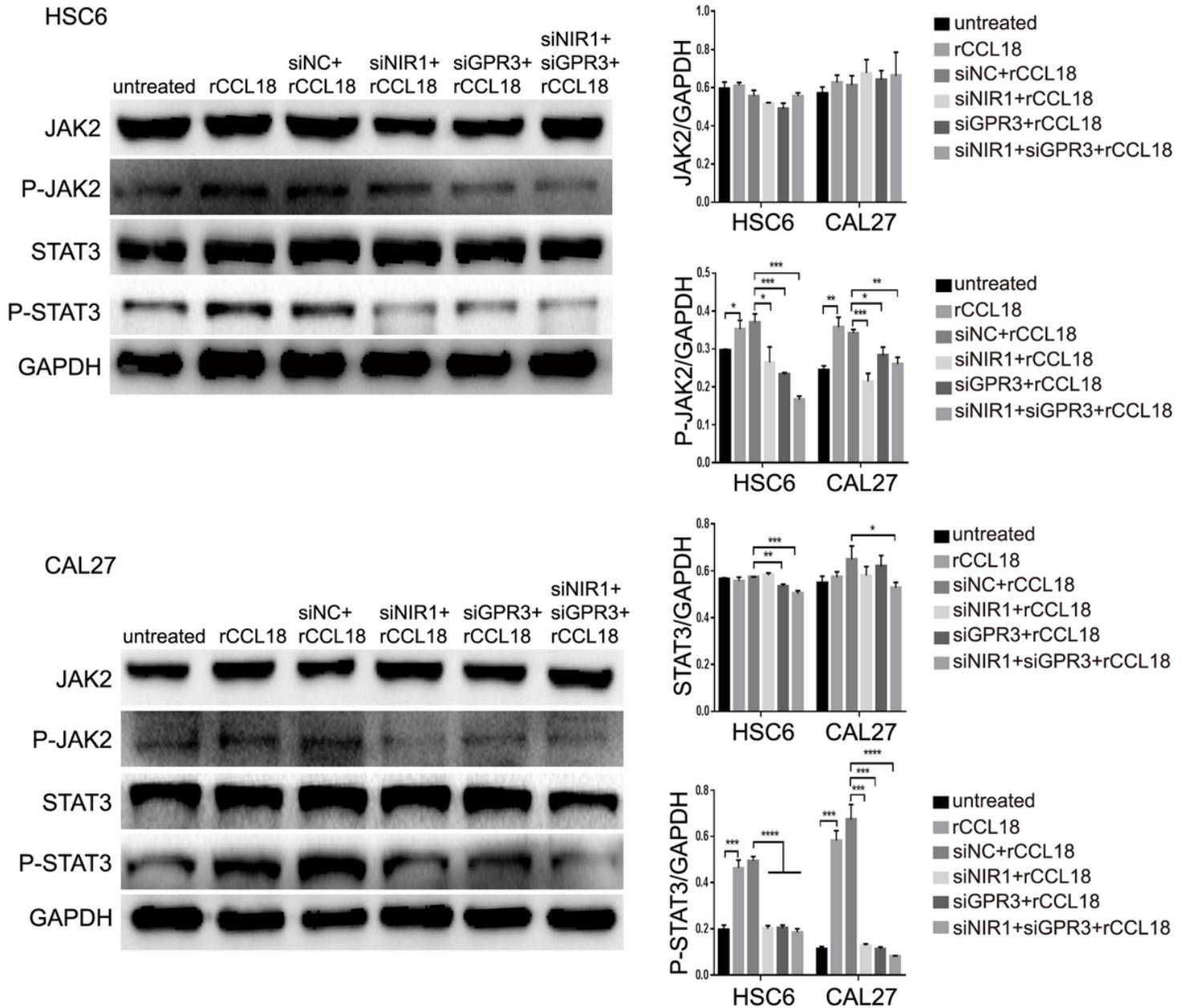


Figure 7

CCL18-NIR1/GPR3 activates the JAK2/STAT3 pathway in OSCC cells. Western blotting showed that the expression of phosphorylated JAK2/STAT3 was appeared in the rCCL18 group and siNC+rCCL18 group, GAPDH was used as internal control. All data are presented as the mean \pm SEM of triplicate experiment. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. control)

Supplementary Files

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