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Effects of CCR7 and Src on invasion and migration of salivary gland tumor

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Abstract. – OBJECTIVE: To explore whether Src activity is regulated by the binding of chemokine receptor 7 (CCR7) and CCL19 in salivary gland tumor. We also aim to elucidate whether Src is capable of regulating invasion and migration of head and neck squamous cell carcinoma (HNSCC) cells.

MATERIALS AND METHODS: PCI-37B cells were first treated with 20 μM PP2 for 30 min or 10 μg/mL CCR7mAb for 4 h, respectively, followed by 200 ng/mL CCL19 induction for 5 min. Western blot was conducted to detect protein levels of p-Src, p-Pyk2 and p-Paxillin. Transwell assay was performed to access migratory and invasive abilities of PCI-37B cells. Immunofluorescence was finally conducted to observe changes in cell cytoskeleton.

RESULTS: CCL19 induction in PCI-37B cells upregulated protein levels of p-Src, p-Pyk2 and p-Paxillin, which were downregulated by PP2 treatment. Src activation induced by CCL19 enhanced invasive and migratory abilities of PCI-37B cells. However, PP2 treatment reversed invasive and migratory abilities even after CCL19 induction. CCL19-induced PCI-37B cells were shaped as irregular polygon and closely connected. Large flak pseudopods were observed and invasive pseudopodia connections markedly increased after CCL19 induction. F-actin body was found in pseudopodia. PP2 treatment resulted in less pseudopodia and regularly arranged actin filaments.

CONCLUSIONS: Src activation is regulated by binding of CCR7 and CCL19 in salivary gland tumor. Activated Src alters cell adhesion ability and cytoskeleton by regulating Pyk2 and Paxillin, thus elevating invasive and migratory abilities of HNSCC cells.

Key Words:
Salivary gland tumor, CCR7, Src, Invasion, Migration.

Introduction

Salivary gland tumor is one of the most common head and neck tumors. It manifests as a high recurrent rate, a high malignant rate and invasive capsule, resulting in poor prognosis and low survival rate[1]. Tumor invasion and migration are extremely complicated processes. Controlling the invasive and migratory processes of malignant tumors is the key in the tumor treatment. The molecular mechanisms of invasion and migration of malignant tumors have been continuously explored in recent years[2-4]. Chemokine receptor 7 (CCR7) is a subfamily of chemokine receptors. CCR7 could affect lymph node metastasis by specifically binding to its corresponding ligands[5-7]. Current researches[8-11] have confirmed the important role of CCR7 in breast cancer, melanoma, non-small cell lung cancer, colon cancer, gastric cancer and head and neck squamous cell carcinoma. Src is widely present in normal cells and cancer cells. Phosphorylated Src stimulates a series of signal transduction pathways or interacts with some important cytokines, thus participating in cell metabolism, development and differentiation. Src is found to be capable of promoting invasion, proliferation and metastasis of tumors. It is reported[12,13] that Src is highly expressed in salivary gland tumors, high invasive head and neck squamous cell carcinoma (HNSCC) and abnormal epithelial cells. Activated Src exerts a vital role in regulating pathogenesis of HNSCC. Src inhibition remarkably inhibits *in vitro* proliferation of tumor cells[14]. Apparently, CCR7 and Src are involved in the regulation of the

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survival, proliferation, migration and invasion of malignant tumor cells, and they also affect the growth and extinction of tumors. However, due to the extremely complex signal transduction pathway of tumors, it is still unclear whether there is a certain correlation between CCR7 and Src. Some studies have suggested that Src can act as an effector of G protein. G protein-coupled receptors activate different signaling pathways and are thought to be the cellular processes of Src regulatory events. During cell transformation, Gai2 and Ga0 regulate G protein-dependent cell proliferation by activating Src and STAT3[15]. Src is considered to be the direct effector of Ga subunit[16]. Napolitani et al[17] showed that activation of Src tyrosine protein kinase in mature dendritic cells requires CCR7 stimulation. However, the interaction between CCR7 and Src in the metastasis and invasion of malignant tumors is rarely reported. In the present study, we aim to explore whether the invasion and migration of salivary gland tumor could be regulated by activated Src after CCR7 binding to CCL19. We also detect protein expressions of adhesion factors and cytoskeleton change in salivary gland tumor cells.

Materials and Methods

Cell Culture

PCI-37B cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 5% CO_2, 37°C. Cell passage was performed every 2-3 days.

Western Blot

Total protein was extracted and loaded in equal amounts. After being separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to the membrane, which was then blocked with 5% skim milk for 1 hour. The specific primary antibody was used to incubate with the membrane overnight at 4°C. After being washed with 1×tris buffered saline and Tween 20 (TBST) for 5 times, the secondary antibody was used to incubate the membrane for 2 h at room temperature. After washing with 1×TBST for 1 min, the chemiluminescent substrate kit was used for exposure the protein band.

Transwell Assay

PCI-37B cells were centrifuged and resuspended in serum-free Dulbecco's Modified Eagle's Medium (DMEM). 20 μL of cell suspension and 500 μL of DMEM containing 0.5% fetal bovine serum (FBS) (500 ng/mL CCL19 was supplied except for those of negative controls) were added in the upper and lower chamber, respectively. After cell culture for 48 h, cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min. Inner cells were carefully cleaned. Migratory cells in 5 randomly selected fields of each sample were captured (magnification 200×). Invasive cells were determined using the same procedures as the above mentioned except for the chamber was pre-coated with 50 μL of diluted Matrigel for 36 h.

Immunofluorescence

PCI-37B cells were divided into three groups as follows: A. PCI-37B cells without any treatment; B. PCI-37B cells were treated with 500 ng/mL CCL19 for 5 min; C. PCI-37B cells were treated with 500 ng/mL CCL19 for 5 min and 20 μM PP2 for 30 min. Cells were harvested for incubating with 50 μL FITC-phalloidin (5 μg/mL) in dark for 30 min. After cell wash, the cytoskeleton was observed and captured using a microscope (magnification 200×).

Statistical Analysis

We used Graphpad Prism (v6.0, La Jolla, CA, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). Differences between two groups were compared using the *t*-test and those among multiple groups were compared using one-way ANOVA, followed by Post-hoc test. $p<0.05$ was considered statistically significant.

Results

Interaction Between CCR7 and Src in HNSCC Cells

Expression level of p-Src in PCI-37B cells was determined by Western blot. It is shown that protein level of p-Src markedly upregulates after CCL19, the specific ligand of CCR7 induction in PCI-37B cells at a dose of 200 ng/mL for 5 min (Figure 1A). Specifically, the level of p-Src in CCL19-induced PCI-37B cells was 1.4 times higher than that of controls. After block-

age the binding of CCR7 and CCL19 by treatment of 10 μg/mL CCR7mAb for 4 h, protein level of p-Src was only 0.8 times of controls. Subsequently, CCL19-induced PCI-37B cells were treated with Src activation inhibitor PP2 (20 μM, 30 min). We found that protein level of p-Src significantly decreases compared with that of controls (Figure 1B). It is concluded that the binding of CCR7 and CCL19 could down-regulate the activation of Src in PCI-37B cells. Blockage the binding of CCR7 and CCL19 reduced protein level of activated Src. CCL19 induction failed to stimulate Src activation in HNSCC cells after PP2 treatment.

Src Activation Induced by CCR7 Inhibition Enhanced Migratory Ability of PCI-37B Cells

Migration of tumor cells is the fundamental key for tumor metastasis. Transwell assay was performed to detect migratory ability of PCI-37B cells. Compared with those of controls, the amount of migratory cells after CCL19 induction was higher, suggesting a stronger migratory ability of PCI-37B cells (Figure 2A and 2B). PCI-37B cells treated with PP2 and CCL19 showed weaker migratory ability compared with those only treated with CCL19 (Figure 2C). Quantification analyses of migratory cells were shown in Figure 2D. The above data elucidated that activated Src by binding of CCL19 and CCR7 enhances migratory ability of PCI-37B cells.

Src Activation Induced by CCL19 Enhanced Invasive Ability of PCI-37B Cells

Invasion is an important feature distinguishing malignant tumor cells from normal cells, which is the leading cause of high mortality in malignant tumors. Transwell chamber was pre-coated with Matrigel to simulate basement membrane. Compared with those of controls, the amount of invasive cells after CCL19 induction was higher, suggesting a stronger invasive ability of PCI-37B cells (Figure 3A and 3B). After treatment of PP2 and CCL19, the invasive ability of PCI-37B cells markedly reduced (Figure 3C). Quantification analyses of invasive cells were shown in Figure 3D. It is elucidated that the invasive ability of PCI-37B cells is enhanced by CCL19, but decreased after Src inhibition.

CCR7 Inhibition Activated Src and Affected Levels of Cell Adhesion Factors in PCI-37B Cells

Through the above experiments, we confirmed the activation of Src by binding of CCR7 and CCL19 in PCI-37B cells, and the subsequent alterations in cell migration and invasion. We speculated that activities of cell adhesion factors are changed during the process. Western blot was conducted to detect protein levels of Pyk2 and Paxillin in PCI-37B cells. The data showed upregulated p-Pyk2 after CCL19 induction, which was 1.4 times higher

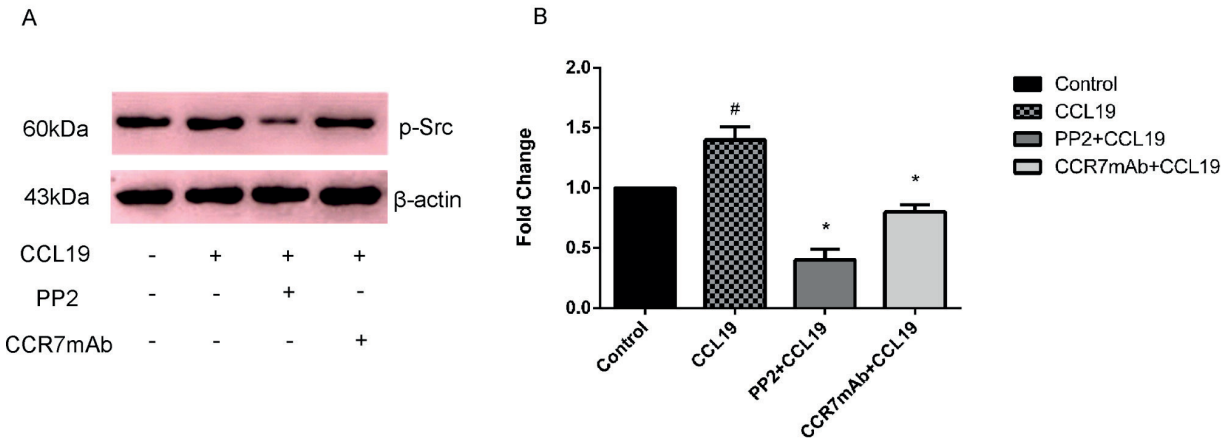

Figure 1. Interaction between CCR7 and Src in PCI-37B cells. PCI-37B cells were treated with 200 ng/mL CCL19 for 5 min, 200 ng/mL CCL19 for 5 min and 20 μM PP2 for 30 min, or 200 ng/mL CCL19 for 5 min and 10 μg/mL CCR7mAb for 4 h, respectively. ***A,*** Protein expression of p-Src in PCI-37B cells was determined by Western blot. ***B,*** Quantification of protein level of p-Src. *$p<0.05$ compared with PCI-37B cells without any treatment; #$p<0.05$ compared with PCI-37B cells treated with CCL19. Each experiment was repeated in triplicate.

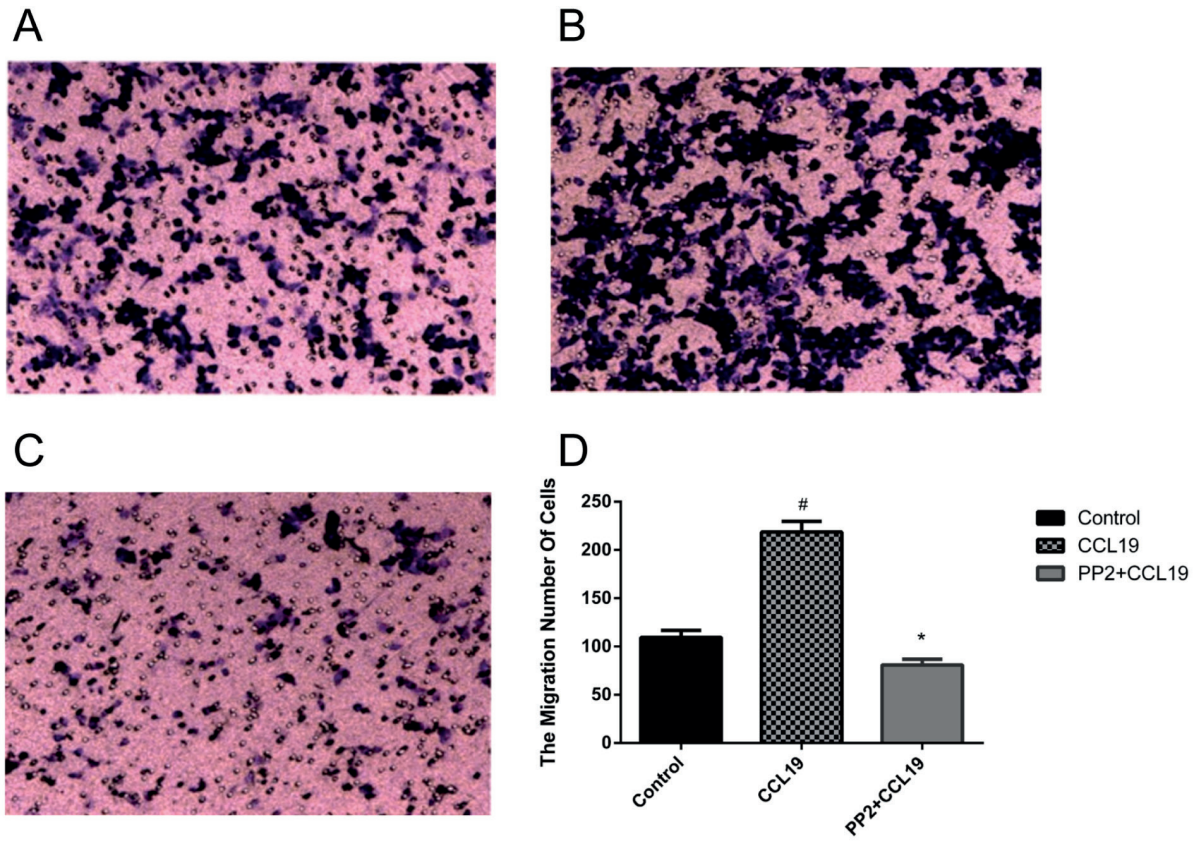

Figure 2. Src activation induced by CCR7 inhibition enhanced migratory abilities of PCI-37B cells. ***A,*** PCI-37B cells without any treatment; ***B,*** PCI-37B cells were treated with 500 ng/mL CCL19 for 5 min; ***C,*** PCI-37B cells were treated with 500 ng/mL CCL19 for 5 min and 20 μM PP2 for 30 min. Transwell assay was conducted to determine the migratory cells (magnification 200×). ***D,*** Quantification of migratory cells in each group. Five randomly selected fields were selected in each group. *$p<0.05$ compared with PCI-37B cells without any treatment; #$p<0.05$ compared with PCI-37B cells treated with CCL19. Each experiment was repeated in triplicate.

than that of controls (Figure 4A). PP2 treatment in CCL19-induced PCI-37B cells significantly decreased p-Pyk2 level (Figure 4B). CCL19 induction also upregulated p-Paxillin level in PCI-37B cells, which increased 1.5 times higher than that of controls (Figure 4C). Similarly, PP2 treatment in CCL19-induced PCI-37B cells downregulated p-Paxillin level as well (Figure 4D).

Src Activation Affected Cytoskeleton of PCI-37B Cells

Changes in cytoskeleton of PCI-37B cells were determined using immunofluorescence as F-actin could stain with FITC-phalloidin. PCI-37B cells without any specific treatment were shaped as irregular polygon or fusiform. A large number of invasive pseudopodia connections could be observed between cells. F-actin presented as a relatively thick bundle of fibers, and some were concentrated around the cells and pseudopods (Figure 5A). After CCL19 induction in PCI-37B cells, they were shaped as irregular polygon and closely connected. Large flak pseudopods were observed and invasive pseudopodia connections markedly increased. Actin filaments were woven into the complex network structure and abundant F-actin formed as F-actin ring or F-actin body (Figure 5B). By comparison, PCI-37B cells treated with PP2 and CCL19 were tended to be round or fusiform. The polar connections between the cells were reduced, and the actin filaments in cells were regularly arranged in parallel (Figure 5C). Through the above observation of cell morphology and F-actin changes, we believed that Src exerts a key role in motility changes of PCI-37B cells.

Discussion

In recent studies on the CCR7 signaling pathway, it has been suggested that the G protein in naive T cells can adhere to inflammatory endothelial cells through Src[18]. Inflammation stimulates the binding of CCR7 to CCL19 or CCL21. Subsequently, Src is phosphorylated by activated CCR7 and an active SH2 binding site is produced, thereby recruiting phosphorylated SHP2 to form a polymer. Hauser et al[19] previously proposed that CCR7 is a tetramer containing two sites that bind to G protein. Src is recruited to form a signaling pathway scaffold that integrates G protein, thereafter regulating expressions of downstream genes. It is concluded that CCR7 is not only regulated by G protein, but also by other signaling pathways. In this study, we found that CCR7 is highly expressed in malignant adenoma PCI-37B cell line (also known as HNSCC cell line). After CCL19 induction, Src was activated and then the binding of CCR7 and CCL19 was blocked. During this process, the expression level of activated Src decreased, suggesting that the activation of Src was closely related to the activation of CCR7. CCR7 can activate Src and downregulate the corresponding signal transduction after binding to its specific ligand CCL1. Although CCR7 was activated after inhibiting the activity of Src protein, the expression of phosphorylated Src did not increase, showing that the activated Src can be inhibited by its own inhibitor. To sum up, we suggest that the activation of Src and CCR7 is closely interacted with each other. High rates of metastasis and invasion in salivary gland tumor are the main causes for its high mortality. Studies on CCR7 and Src in regulating the invasion and migration of salivary gland tumor cells have been reported. It is suggested that CCR7 can activate the small G protein Rho, thereby activating Pyk2 and the downstream signaling factor cofilin, finally af-

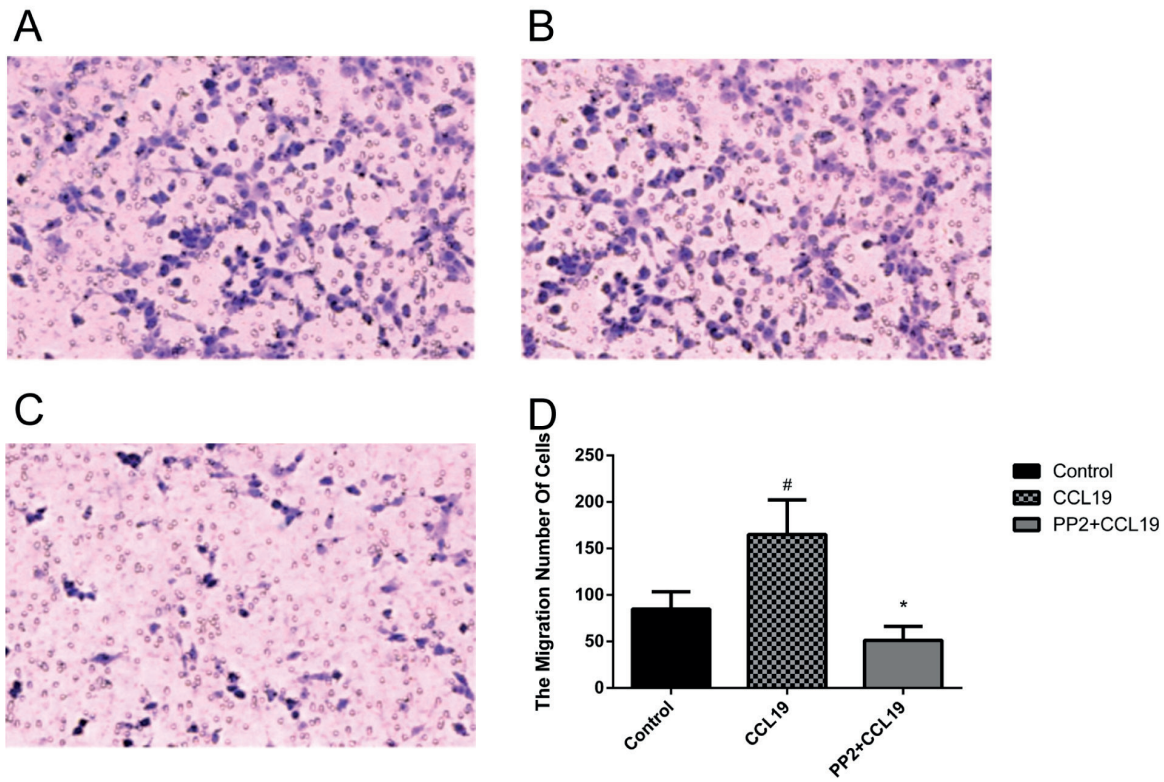

Figure 3. Src activation induced by CCR7 inhibition enhanced invasive abilities of PCI-37B cells. ***A,*** PCI-37B cells without any treatment; ***B,*** PCI-37B cells were treated with 500 ng/mL CCL19 for 5 min; ***C,*** PCI-37B cells were treated with 500 ng/mL CCL19 for 5 min and 20 μM PP2 for 30 min. Transwell assay was conducted to determine the invasive cells (magnification 200×). ***D,*** Quantification of invasive cells in each group. Five randomly selected fields were selected in each group. *$p<0.05$ compared with PCI-37B cells without any treatment; #$p<0.05$ compared with PCI-37B cells treated with CCL19. Each experiment was repeated in triplicate.

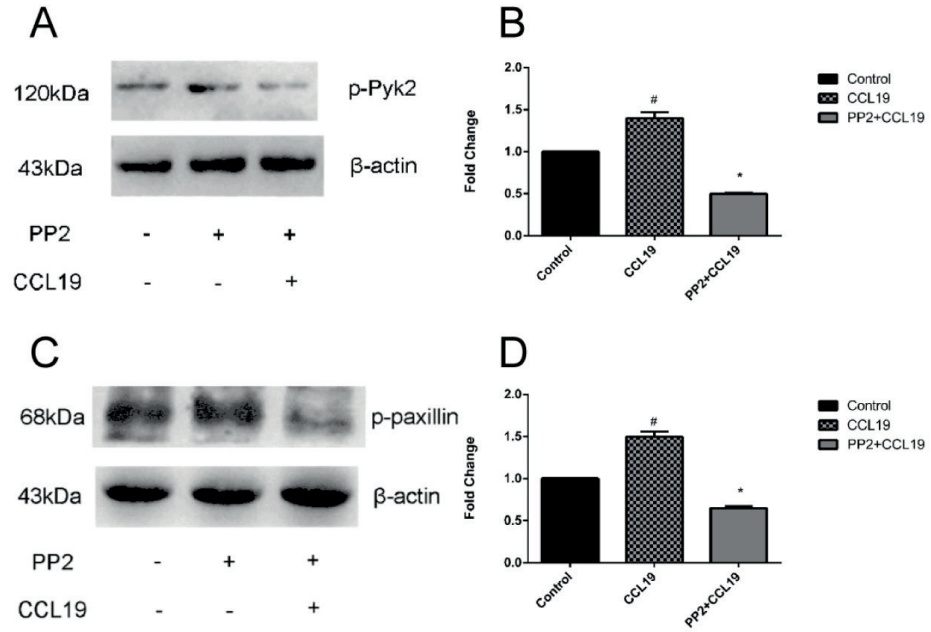

Figure 4. CCR7 inhibition activated Src and affected levels of cell adhesion factors in PCI-37B cells. PCI-37B cells were treated with 200 ng/mL CCL19 for 5 min, followed by treatment with or without 20 μM PP2 for 30 min. ***A,*** Protein level of p-Pyk2. ***B,*** Quantification of protein level of p-Pyk2. ***C,*** Protein level of p-Paxillin. D. Quantification of protein level of p-Paxillin. *$p<0.05$ compared with PCI-37B cells without any treatment; #$p<0.05$ compared with PCI-37B cells treated with CCL19. Each experiment was repeated in triplicate.

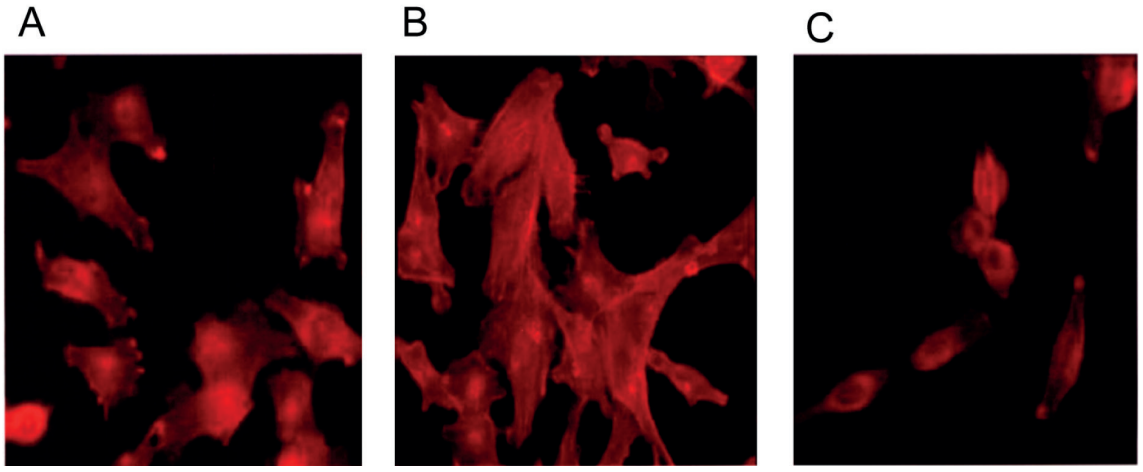

Figure 5. Src activation affected cytoskeleton of HNSCC cells. ***A,*** PCI-37B cells without any treatment; ***B,*** PCI-37B cells were treated with 500 ng/mL CCL19 for 5 min; ***C,*** PCI-37B cells were treated with 500 ng/mL CCL19 for 5 min and 20 μM PP2 for 30 min. Immunofluorescence was performed to observe cytoskeleton of PCI-37B cells (magnification 200×).

fecting the migration and invasion of salivary gland tumor cells[20-22]. Src regulates invasion and migration of salivary gland tumor cells by stimulating MAPK pathway and small G protein Rho[23,24]. However, whether Src is interacted with CCR, thus involving in the regulation of invasion and migration of salivary gland tumor cells, remains unclear. In the present study, the migratory and invasive abilities of PCI-37B cells after CCL19 induction were determined by transwell assay. The data showed that the activated Src by CCL19 induction enhanced migratory and

invasive abilities of PCI-37B cells. We suggest that CCR7 could interact with Src, and subsequently affect the invasive and migratory abilities of PCI-37B cells. We further investigated the potential mechanism in the regulation of invasion and migration of PCI-37B cells. Relative researches have pointed out that cell adhesion and cytoskeletal changes directly affect the metastasis of malignant tumors[25]. In view of the importance of Pyk2 and Paxillin in migration and invasion of tumor cells, Western blot was conducted to detect their protein expressions in PCI-37B cells. After Src activation by CCR7 binding to CCL19, phosphorylation and dephosphorylation of Src remarkably regulated the activities of Pyk2 and Paxillin in salivary gland tumor cells. Furthermore, we observed the cytoskeleton and cell morphology of PCI-37B cells by immunofluorescence assay. It was found that the activation of Src induced by CCL19 could change the cell bone architecture and affect the formation of invasive pseudopods, thereby regulating migration and invasion of PCI-37B cells. In summary, we suggest that there is a close relationship between CCR7 and Src in salivary gland tumor cells. We suggested that CCR7 binds to its specific ligand CCL19, recruits and activates Src to form a stable signaling pathway molecular structure. Pyk2 and Paxillin are then stimulated and lead to changes in cytoskeleton and cell adherent ability, finally regulating invasion and migration of salivary gland tumor cells. We found that after blocking the binding of CCR7 to CCL19, the expression of activated Src decreased, but the expression of p-Src was upregulated. The specific interaction mechanism between CCR7 and Src in salivary gland tumor still remains unclear. Whether there is an important target that could block the downstream of CCR7 and Src at the same time is unknown, which requires further explorations.

Conclusions

We indicated that Src activation is regulated by binding of CCR7 and CCL19 in salivary gland tumor. Activated Src alters cell adhesion ability and cytoskeleton by regulating Pyk2 and Paxillin, thus elevating invasive and migratory abilities of salivary gland tumor cells.

Conflict of interest

The authors declare no conflicts of interest.

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