

# Chemokine receptor 7 via proline-rich tyrosine kinase-2 upregulates the chemotaxis and migration ability of squamous cell carcinoma of the head and neck

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**Abstract.** Aberrant regulation in the chemotaxis and migration ability of cancer cells is closely associated with their metastatic activity. The chemokine receptor 7 (CCR7) has recently been shown to play an important role in regional lymph node metastasis of squamous cell carcinoma of the head and neck (SCCHN). In this study, we examined the role of proline-rich tyrosine kinase-2 (Pyk2) in CCR7-induced chemotaxis and migration ability of metastatic SCCHN cells. We showed that Pyk2 is overexpressed in squamous cell carcinoma of the head and neck. We also found that CCR7 induced Pyk2 and cofilin activation. Inhibition of Pyk2 activity using a pharmacological inhibitor, Tyrphostin A9, significantly attenuated CCR7-induced Pyk2 tyrosine phosphorylation, activation of cofilin and sequentially abolished F-actin rearrangement, diminished the chemotaxis and migration ability of SCCHN cells. In summary, our data suggest that CCR7 via Pyk2 and cofilin regulates the chemotaxis and migration ability of metastatic SCCHN cells.

## Introduction

The 5-year survival for patients with squamous cell carcinoma of the head and neck is only 30%, mainly due to the frequent presence of metastasis at diagnosis (1). The pattern of regional cervical metastasis in this disease is remarkably consistent. A better understanding of this unique metastatic process is necessary to enable the development of therapies designed to prevent tumor dissemination.

Chemokines and their respective receptor that regulate chemotaxis and the transendothelial migration of leukocytes during immune and inflammatory reactions were recently

observed to play an important role in the metastasis of various types of cancer (2,3). Chemokine receptors have been found to be expressed in these invasive cells in a distinct and non-random pattern. In particular, metastatic SCCHN cells have been shown to express chemokine receptor 7 (CCR7), which may enable their access to the lymphatic system and facilitate spread to regional lymph nodes (4-7). However, the signaling mechanisms mediated by CCR7 and induced by CCL19 have yet to be elucidated in SCCHN cells.

Proline-rich kinase-2 (Pyk2) is a non-receptor protein tyrosine kinase structurally related to focal adhesion kinase. Pyk2 has a restricted tissue expression primarily in neuronal and hematopoietic tissues. The tyrosine 402 (Y402) at Pyk2 serves as the primary autophosphorylation site that is essential for Pyk2 activity and function (8). Pyk2 is rapidly activated by G-protein coupled receptor agonists, growth factors, cytokines and stress signals, thus regulating various functions such as cell adhesion, migration, proliferation and survival (9,10). Pyk2 is also involved in regulating the migration and invasion of human glioma, prostate and breast cancer cells (11-13). It has previously been demonstrated that the activation of Pyk2 by CCR7 was involved in dendritic cells (DC) (14). Therefore, we hypothesized that Pyk2 might be involved in CCR7 mediated signals in SCCHN cells. Our results showed that CCL19 induced the activation of Pyk2 and the activation of cofilin which altered actin cytoskeletal rearrangement, and this signal pathway was crucial for the chemotaxis and migration of SCCHN cells.

## Materials and methods

**Cell line and human tumor samples.** The metastatic SCCHN cell line PCI-37B, which strongly expresses CCR7, was a kind gift from the University of Pittsburgh. Cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA, USA), which contained 10% (v/v) heat-inactivated fetal bovine serum (Gibco-BRL Corp., Grand Island, NY, USA), 100 U/ml penicillin and 100 U/ml streptomycin.

Sixty specimens of SCCHN tumors with the adjacent metastatic (or normal) lymph nodes and 10 specimens of normal human oral mucosal tissue were obtained from the Head and Neck Tumor Center, School of Stomatology, China Medical University. All the specimens were obtained with the

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Table I. Correlation between Pyk2 expression and clinicopathological factors of SCCHN.

Clinicopathological characteristics	No. of cases	+ to +++	-	Statistical analysis ( $\chi^2$ test)
Age (years)				
$\geq 60$	36	20	16	0.049
<60	24	15	9	
Tumor size				
T1, T2	49	28	21	0.156
T3, T4	11	7	4	
Clinical stage				
I, II	28	11	17	7.837 <sup>a</sup>
III, IV	32	24	8	
Nodal metastasis				
No	30	11	19	11.589 <sup>a</sup>
Yes	30	24	6	

<sup>a</sup>P<0.05 (the internal difference of Pyk2 expression within clinicopathological characteristics).

consent of the patients before surgery and in accordance with Health Insurance Portability. The classification of SCCHN, including primary tumors (T), regional lymph nodes (N), distant metastasis (M) and stage grouping, was determined according to the rules of the International Union Against Cancer (UICC) for Head and Neck Cancer (tumor node metastasis, TNM classification, 1997).

**Reagents and antibodies.** The CCR7 chemokine ligand (CCL19, MIP-3 $\beta$ ) and the anti-hCCR7 mAb were purchased from R&D Systems (Minneapolis, MN, USA). The Pyk2 inhibitor (Tyrphostin A9) was purchased from Calbiochem (San Diego, CA, USA). Anti-Pyk2, anti-phospho-Pyk2(402) and anti-p-cofilin were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TRITC-labelled phalloidin was from Sigma (St. Louis, MO, USA).

**Chemotaxis assay.** Chemotaxis in response to chemokine was determined by measuring the number of cells migrating through a polycarbonate filter (8- $\mu$ m pore size) in 24-well transwell chambers in triplicate in DMEM with 0.5% (w/v) BSA (Invitrogen). The cell suspensions (2x10<sup>5</sup> cell/200  $\mu$ l), were placed in the top chamber of the filter. Aliquots of the chemokine were added to the wells. After 24 h, cells in each lower well were counted under a light microscope in at least five different fields (original magnification, x200). The mean  $\pm$  SD was recorded for each condition, and index calculated based on the control, random migration.

**Matrigel invasion assay.** Cell invasion was quantified *in vitro* using Matrigel-coated semipermeable, modified inserts with a pore size of 8  $\mu$ m. The analysis of invasion assay was performed as described in the chemotaxis assay incubated with CCL19 for 36 h.

**Western blotting.** Briefly, 70-80% confluent cells were serum-starved for 24 h. Following treatment, cells were lysed in M-PER

reagent (Pierce) containing 1 mM PMSF and phosphatase inhibitors and centrifuged at 4°C, 12,000 rpm for 30 min. The supernatant protein was normalized and 80  $\mu$ g of protein was size-fractionated and immunoblotted with the indicated mAbs, we determined Pyk2, p-Pyk2(402) and p-cofilin protein expression.

**Actin polymerization assay.** PCI-37B cells pretreated with/without CCR7 mAb and Pyk2 inhibitor Tyrphostin A9 were fixed, permeabilized and stained with TRITC-labeled phalloidin. Following labeling, the samples were washed three times for 10 min each in PBS to remove the unincorporated label. F-actin distribution following CCL19 stimulation was evaluated by confocal laser scanning microscope (CLSM, Leica SP2, Germany).

**Immunohistochemical analysis.** Immunohistochemical staining used conventional horseradish peroxidase immunohistochemical staining methods. In brief, 5- $\mu$ m sections of the specimens were deparaffinized and hydrated with 0.6% H<sub>2</sub>O<sub>2</sub> in methanol to inhibit endogenous peroxidase, performed antigen retrieval and incubated with normal blocking serum for 10 min. The sections were then incubated with primary antibodies (1:100): rabbit anti-Pyk2 polyclonal antibody overnight at 4°C. Immunodetection was performed using peroxidase labeled secondary antibody (R&D Systems) and diaminobenzidine for visualization. All the sections were counterstained with hematoxylin (Sigma). Negative controls included omission of the primary antibody. The cell morphology was analyzed by microscopy (Nikon Eclipse 80i, Tokyo, Japan) at x100-400 magnification. According to the percentage of positive tumor cells, all these cells were scored as negative (-), <10% or no staining; weak positive (+), 11-50%; positive (++), 51-75%; or strongly positive (+++), >75%.

**Statistical analysis.** Numerical data were expressed as the mean  $\pm$  standard deviation (SD). Statistical differences between two groups were evaluated using Student's t-test. Correlation

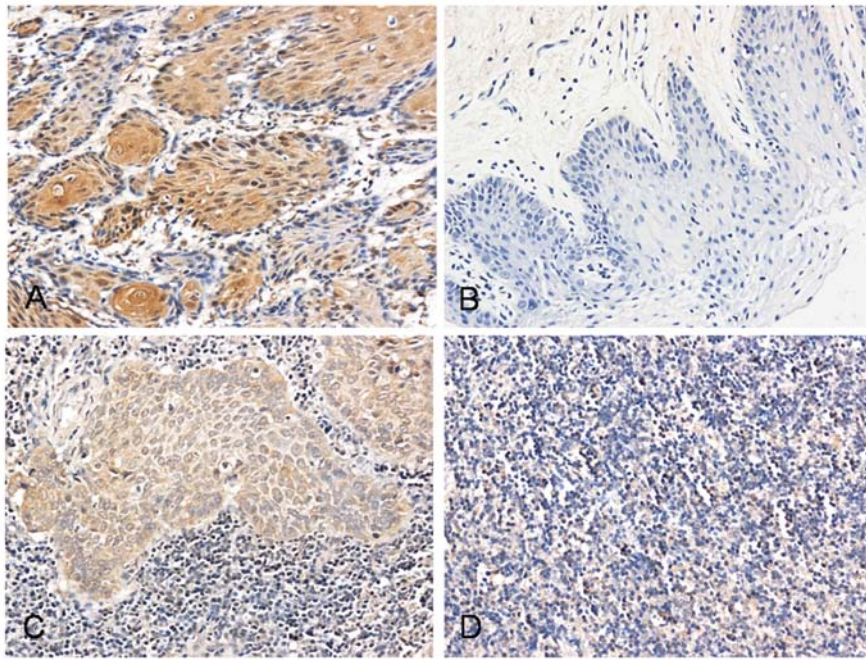


Figure 1. Immunohistochemical staining of Pyk2 in (A) primary SCCHN, (B) normal tissues, (C) metastatic lymph nodes and (D) normal lymph nodes (original magnification, x200).

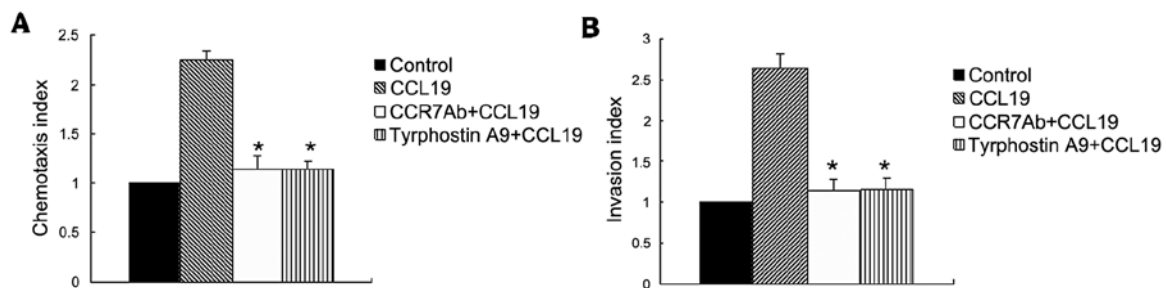


Figure 2. Tyrphostin A9 diminished the CCR7-induced chemotaxis and invasion ability of PCI-37B cells. PCI-37B cells were pretreated with CCR7 Ab (10  $\mu$ g/ml, at 37°C for 4 h) or Pyk2 inhibitor Tyrphostin A9 (10  $\mu$ M, at 37°C for 1 h), then followed by CCR7 ligand, CCL19 (500 ng/ml), as a chemoattractant and DMEM media alone as the blank control. (A) Chemotaxis assay chambers were incubated at 37°C for 24 h. (B) *In vitro* invasion assay chambers were incubated at 37°C for 36 h. Results are shown as the mean  $\pm$  SD n=3, \*P<0.01. Comparison of treatment of cells with CCR7 Ab or Tyrphostin A9 to treatment of cells with CCL19 alone.

between Pyk2 expression and SCCHN clinical stage was analyzed by  $\chi^2$  test. Values of P<0.05 were considered to indicate statistically significant differences. All statistical analyses were performed with the software SPSS 13.0.

## Results

*Pyk2 is overexpressed in squamous cell carcinoma of the head and neck.* Using immunohistochemistry, we investigated the expression of Pyk2 in SCCHN tumor tissues, metastatic lymph nodes, normal lymph nodes and oral mucosal tissues. Pyk2 was found in the cell membrane and cytoplasm in tumor cells and lymph node metastatic cells. The number of stained cells was low or absent in normal lymph nodes and oral mucosal tissues (Fig. 1 and Table I). The expression of Pyk2 was significantly correlated with cervical lymph node metastasis and SCCHN clinical stage (P<0.05)

*Correlation of the migration ability and the activities of CCR7 and Pyk2 in the metastatic SCCHN cell line.* To investigate the molecular mechanism that regulates the chemotaxis and migration ability of SCCHN, we utilized the metastatic tumor cell line (PCI-37B) which expresses CCR7, then analysed their capability to migrate *in vitro* in response to the respective chemokine ligand CCL19. This experiment showed that CCL19 enhanced chemotaxis of SCCHN significantly as compared with background control levels established with media alone. The Pyk2 inhibitor and anti-CCR7 mAb significantly blocked CCL19-induced cell chemotaxis, as shown in Fig. 2A.

In addition to chemotactic ability, we evaluated the invasive capacity mediated by CCR7 in the metastatic SCCHN cell line. *In vitro* invasion through Matrigel was assessed after exposure of these cells to the CCR7 ligand, CCL19, in the presence or absence of Tyrphostin A9 or pretreatment with a CCR7-specific blocking mAb. Fig. 2B shows that treatment of

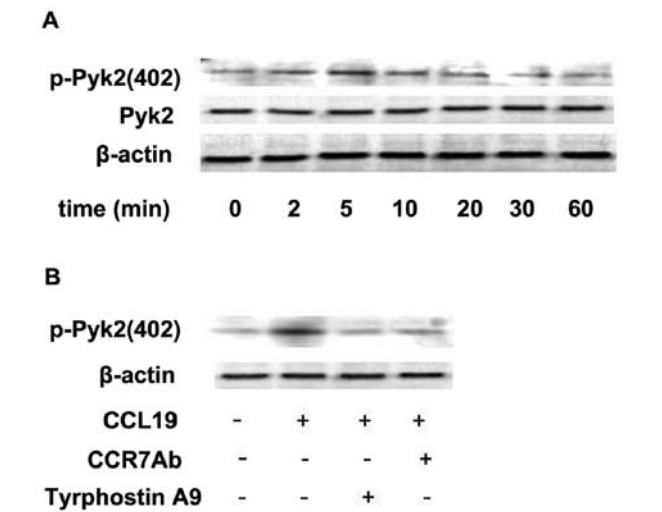


Figure 3. CCR7 stimulated activation of the tyrosine kinase Pyk2. (A) Time course of CCL19-stimulated Pyk2 activity in PCI-37B cells. PCI-37B cells were stimulated for the indicated times with 200 ng/ml CCL19. (B) Effect of CCR7 Ab on CCL19-induced Pyk2 activations in PCI-37B cells. PCI-37B cells pretreated with/without Tyrphostin A9 (10 μM, at 37°C for 1 h) or CCR7 Ab (10 μg/ml, at 37°C for 4 h) were stimulated with 200 ng/ml CCL19 for 5 min.

cells with tyrphostin A9 significantly abolished the CCL19-induced invasive capacity ( $P<0.01$ ), indicating the invasive capacity mediated by CCR7 required Pyk2 activity.

**Role of CCR7 in regulating the Pyk2 activity in metastatic SCCHN.** Since tyrosine kinase Pyk2 has been shown to be involved in the signaling from chemokine receptors (11), we examined whether stimulation of metastatic SCCHN cells with CCL19 modulates the activity of this kinase. The metastatic SCCHN cell line (PCI-37B) was stimulated with chemokines for different time periods. We found that CCL19 induced a transient increase in the activity of Pyk2 (Fig. 3A). Autophosphorylation of Pyk2 increased as early as 2 min after the addition of chemokine to the cells, reached a maximum after 5-10 min and returned to basal levels after 60 min (Fig. 3A). To define the role of CCR7 mAb in regulating Pyk2 activity we examined the activity of this kinase in the cells pretreated with/without CCR7 mAb and Pyk2 inhibitor Tyrphostin A9. As shown in Fig. 3B, CCL19 induced Pyk2 activation to a level 2- to 3-fold above the baseline, which was determined by incubation with media alone. Phosphorylation of this molecule was blocked selectively by the Pyk2 inhibitor

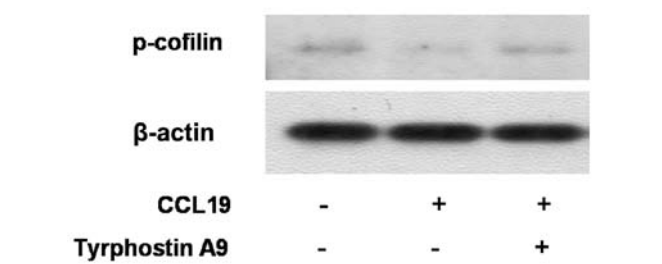


Figure 4. CCL19-induced cofilin activations were blocked by Pyk2 inhibitor in PCI-37B cells. PCI-37B cells pretreated with/without Tyrphostin A9 (10 μM, at 37°C for 1 h) were stimulated with CCL19 (200 ng/ml, at 37°C for 5 min).

(Tyrphostin A9) and CCR7 mAb. These experiments were repeated at least three times with similar results.

**CCR7 inhibits phosphorylation/inactivation of cofilin that is mediated by Pyk2.** Cofilin mediates lamellipodium extension and polarized cell migration by stimulating actin filament dynamics at the leading edge of migrating cells. Cofilin is inactivated by phosphorylation at Ser-3. We analyzed whether cofilin is downstream of Pyk2. The western blot experiment showed that CCL19 inhibited phosphorylation/inactivation of cofilin in SCCHN cells. Markedly, Tyrphostin A9 blunted the decrease in phosphorylation of cofilin (Fig. 4), indicating that cofilin is downstream of Pyk2. In summary, the result indicates that CCR7 inhibits phosphorylation/inactivation of cofilin that is mediated by Pyk2.

**CCR7 induces F-actin rearrangement.** Cell motility involves regulation of the actin cytoskeleton and the actin-severing protein cofilin regulates actin organization. We found that CCR7 activation lead F-actin polymerization and pseudopodia formation. In untreated cells we observed a scattered distribution of F-actin (Fig. 5). In the cells treated with CCL19 F-actin arrays and pseudopodia were formatted, while these effects were blocked by Tyrphostin A9. We therefore consider that the actin cytoskeletal rearrangement induced by CCL19 requires Pyk2.

Discussion

The chemotaxis and migration ability of tumor cells plays a critical role for successful metastasis (14). CCR7 has been shown to be involved in the metastasis of SCCHN (4), and

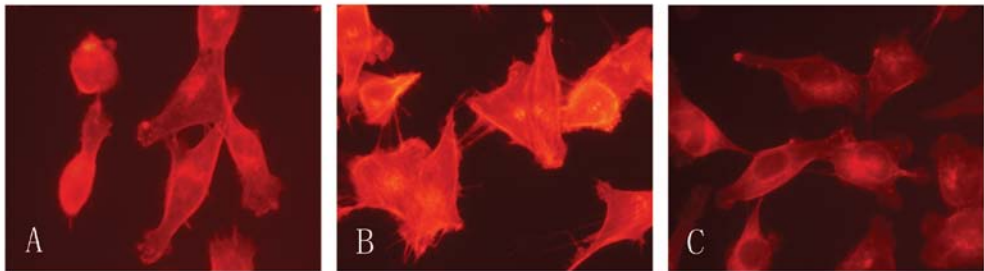


Figure 5. CCL19-induced and Tyrphostin A9 blocked reorganization of the actin cytoskeleton of PCI-37B cells. PCI-37B cells were (A) untreated, (B) treated with CCL19 (500 ng/ml, for 0.5 h), or (C) treated with Tyrphostin A9 (10 μM, for 1 h) followed by CCL19 (500 ng/ml for 0.5 h). The cells were stained by Rhodamine-labeled phalloidin.

previous studies have presented a picture of the function of this gene (15-18). However, the exact role of CCR7 in tumor progression and its underlying mechanism remain unclear. Pyk2 is also involved in regulating the migration and invasion of a variety of different tumor types. ErbB-2 via Pyk2 upregulates the adhesive ability of androgen receptor-positive human prostate cancer cells (19). Pyk2 has been found to be crucial to cell motility and SOCS3 can regulate Pyk2 pro-migratory function in lung cancer (20). In breast cancer cells, Pyk2 participates in CXCR4-induced chemotactic and chemoinvasive signaling pathways (21). However, there is no report on the role of Pyk2 in SCCHN. Our experiments showed that Pyk2 is highly expressed in SCCHN and metastatic lymph node cells. The expression of Pyk2 was significantly correlated with cervical lymph node metastasis and SCCHN clinical stage. Hence, we considered that Pyk2 is probably crucial for the development and progress of SCCHN.

Tyrosine kinase Pyk2 has been involved in the signaling from chemokine receptors in different cells. In breast cancer cells, CXCL12 induces the phosphorylation at residues 402 and 579/580 of Pyk2, Pyk2 inhibitors significantly blocks CXCL12-induced chemotaxis and chemoinvasion and so the role of Pyk2 is indicated in CXCL12-induced breast cancer cell migration and invasion (22). In T cells, Pyk2 becomes activated in response to chemokine receptors and is important for cell spreading and migration (23). Some results indicate that Pyk2 acts as a receptor-proximal link between integrin and chemokine receptor signaling, and the Pyk2/Rac pathway plays a pivotal role in the control of NK cell transendothelial migration (24). We examined the role of Pyk2 in CCR7-induced metastasis of SCCHN cells. We found that CCL19 (CCR7 ligand) induced the activation of Pyk2 in metastatic SCCHN cells and the activation was blocked by CCR7 mAb. Then, we used the Pyk2 inhibitor, Tyrphostin A9, to analyze the role of Pyk2 in the chemotaxis and migration of SCCHN cells. The results revealed the predominant role of CCR7 in upregulating the Pyk2 activity which was important for the chemotaxis and migration ability of SCCHN cells. Further experiments are required to clarify if other molecules are involved in the CCR7/Pyk2 pathway.

The actin severing protein cofilin is essential for directing cell migration and chemotaxis in many cell types and is also important for tumor cell invasion during metastasis (25,26). Through its severing activity, cofilin increases the number of free barbed ends to initiate actin polymerization for actin-based protrusion. In this regard, we notably observed that stimulation of CCR7 induced dephosphorylation/activation of cofilin. The Tyrphostin A9 abolished the activation of cofilin, suggesting that Pyk2 is upstream of this molecule. Our results indicate that cofilin may mediate the effects of Pyk2 on cell motility.

A high level of actin polymerization is required for the formation of pseudopodia, which is needed for chemokine-mediated cell migration and invasion into surrounding tissues and efficient metastasis formation (27). In our study, we examined rhodamine-labeled phalloidin staining by inverted microscopy, and observed reorganization of the actin cytoskeleton of PCI-37B was enhanced in response to CCL19, and this function was inhibited by Tyrphostin A9. This result, consistent with previous studies in hepatocellular carcinoma, demonstrates that Pyk2 upregulates the formation of pseudo-

podia and actin stress fiber polymerization of HCC cells and promotes cell motility (28).

In conclusion, we found that CCR7 promoted tumor metastasis by sequential activation of Pyk2 and cofilin followed by rearrangement of F-actin in SCCHN cells. Our results thus reveal a novel signal pathway that regulates the chemotaxis and migration ability of SCCHN cells and provide potential targets for advanced therapy of squamous cell carcinoma of the head and neck.

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