Chemokine (C-C motif) ligand 21/C-C chemokine receptor type 7 triggers migration and invasion of human lung cancer cells by epithelial-mesenchymal transition via the extracellular signal-regulated kinase signaling pathway

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Abstract. C-C chemokine receptor type 7 (CCR7) has been implicated in lymph node metastasis of various cancers. Previous studies have revealed that epithelial-mesenchymal transition (EMT) is involved in the chemotactic process mediated by CCR7 and its ligands in various types of carcinoma. However, the underlying mechanism of this process remains to be fully elucidated. The present study investigated whether chemokine (C-C motif) ligand 21 (CCL21)/CCR7 may activate EMT of lung cancer cells and their associated signaling pathways. A549 and H520 lung cancer cell lines were examined in vitro in the present study. The results indicated that A549 and H520 expressed CCR7, but reduced levels of CCL21. Following stimulation of lung cancer cell lines with CCL21, the expression of the epithelial marker E-cadherin was downregulated, and the mesenchymal markers Vimentin/Slug and extracellular signal-regulated kinase (ERK) were upregulated. In addition, the ERK inhibitor PD98059 may inhibit EMT caused by CCL21, and decreased cell migration and invasion initiated by CCL21. Furthermore, lung adenocarcinoma tissues from 50 patients who underwent lung cancer operations were investigated by immunohistochemistry. The findings revealed that CCR7, Slug and Vimentin were highly expressed in lung carcinoma tissues, and were significantly associated with lymph node metastasis and clinical pathological stages, respectively. CCR7 expression was correlated positively with expression levels of Slug and Vimentin. CCL21 was expressed positively in the endothelium of lymphatic vessels adjacent to cancer cells, and weakly in lung cancer cells. Collectively, these results demonstrated that CCL21/CCR7 may activate EMT in lung cancer cells via the ERK1/2 signaling pathway. The current study provides evidence that a close interaction exists between CCL21/CCR7 chemotaxis and EMT procedures in lung cancer metastasis, providing a basis for the development of therapeutic targets.

Introduction

Epithelial-mesenchymal transition (EMT) and chemotaxis are critical processes in cancer metastasis (1-3), and their interaction in cancer metastasis has been investigated in recent years. Cancer cells acquire the capability to detach and migrate away from primary tumors by EMT, involving loss of an epithelial phenotype, including E-cadherin, and gain of a mesenchymal phenotype, including Vimentin and Slug (1,2). Chemokine and receptor interactions guide cancer cells to migrate to distance favorite organs or lymph nodes (3).

Previous studies have identified that certain chemokine receptors, including C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 7 (CCR7), have been detected in a variety of cancer cells and were driven by their ligands to targeted metastasis of cancers (3,4). CCR7 is shared by its ligands chemokine (C-C motif) ligand (CCL) 19 or CCL21, which are produced specifically in secondary lymphatic tissues, including lymph nodes, Peyer's patches and splenic follicles (5-8). Therefore, CCR7 is considered to contribute to
lymph node metastasis of cancers (9,10). Many growth factors, including transforming growth factor (TGf)-β, basic fibroblast growth factor and platelet-derived growth factor have been identified as EMT inducers, and the molecular mechanisms underlying promotion of cancer cell invasion have been investigated (1). Notably, previous studies have revealed that CXCR4 (11,12), CCR2 (13) and CCR6 (14) binding with their chemokines may induce EMT in cancer cells to stimulate cancer cell mobility. Recently, CCL21/CCR7 were demonstrated to activate EMT in human breast cancer cells (15), and subsequently, other studies reported that CCL21/CCR7 may activate the EMT process in gastric cancer cells (16).

Lung carcinoma is one of the most lethal malignancies with poor prognosis due to its ability to metastasize, causing ~84,590 mortalities in men and ~71,280 mortalities in women in the United States in 2016 (17). However, the role of CCR7 and its ligands in EMT of human lung cancer cells remains to be fully clarified. The present study investigated the capacity of CCL21/CCR7 for stimulating EMT, and associated extra-cellular signal-regulated kinase (ERK) 1/2 signaling in lung cancer.

Materials and methods

Cell culture and reagents. The NCI-H157, A549, 973 and NCI-H520 human lung carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO2. Protein expression levels of CCR7 and CCL21 in these cells were detected by western blot analysis, and the results indicated that CCR7 expression levels were differential in A549 and H520 cells; therefore, they were selected for further analysis. A549 and NCI-H520 cell lines were treated with or without CCL21 (cat. no. 300-35; PeproTech, Rocky Hill, NJ, USA) at designed concentrations and times. PD98059 (cat. no. 9900; Cell Signaling Technology, Inc.) and anti-ERK1/2 (dilution 1:1,000; cat. no. ZB-5301; ZSGB-Bio, Beijing, China) were used at room temperature for 1 h for the detection of immunoreactive proteins by a luminochemiluminescence reagent (BioRad). GAPDH. The PCR conditions were: Initial denaturation at 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing at 60°C for 30 sec. Experiments were performed in triplicate.

Western blot analysis. The total protein was extracted from the cultured cells using the Beyotime lysis buffer containing radioimmunoprecipitation buffer and a protease inhibitor cocktail (Beyotime Institute of Biotechnology, Haimen, China) followed by centrifugation in 12,000 x g at 4°C for 30 min. A total of 50-60 µg of protein lysates were separated by 10% SDS-PAGE and subsequently transferred onto polyvinylidene fluoride membranes (PVDF; EMD Millipore, Billerica, MA, USA), followed by blocking with 5% bovine serum albumin (Amresco, LLC, Solon, OH, USA) in TBS with Tween-20 for 2 h. The PVDF membranes were incubated with the following primary antibodies: Anti-β-actin (dilution, 1:2,000; cat. no. 8457P; Cell Signaling Technology, Inc.), anti-CCR7 (dilution, 1:5,000; cat. no. ab32527; Abcam, Cambridge, UK), anti-CCCL21 (dilution, 1:400; cat. no. sc-5808; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Slug (dilution, 1:400; cat. no. sc-166476; Santa Cruz Biotechnology, Inc.), anti-E-cadherin (dilution, 1:1,000; cat. no. ab133597; Abcam), anti-Vimentin (dilution, 1:1,000; cat. no. 5741P; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-ERK1/2 (dilution 1:1,000; cat. no. 4370; Cell Signaling Technology, Inc.) and anti-ERK1/2 (dilution 1:1,000; cat. no. 4695; Cell Signaling Technology, Inc.) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies, including goat anti-mouse IgG (dilution, 1:10,000; cat. no. ZB-5305; ZSGB-Bio, Beijing, China), goat anti-rabbit IgG (dilution, 1:10,000; cat. no. ZB-5301; ZSGB-Bio) and rabbit anti-goat IgG (dilution, 1:10,000; cat. no. ZB-2306; ZSGB-Bio) were used at room temperature for 1 h for the detection of immunoreactive proteins by a luminchemiluminescence reagent (Santa Cruz Biotechnology, Inc.). The semi-quantitative analyses of data were carried out using ImageJ (version, 2.1; Windows 7; National Institutes of Health, Bethesda, MD, USA). The experiment was performed three times.

Wound healing assay. A549 and NCI-H520 cells (3x104) were seeded in 6-well culture dishes and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), incubated with or without 50 µM PD98059 (cat. no. 9900; Cell Signaling Technology, Inc.) at 37°C for 1 h, and subsequently treated with 100 ng/ml CCL21 (cat. no. 300-35; PeproTech). When cells reached confluence, a scratch in the central area was established by a sterile pipette tip, followed by washing with PBS to remove cell debris and replenishing with fresh medium. Following incubation for 48 h, the migrated cells in the wound space were counted under a phase-contrast light microscope. The experiment was repeated three times.
Matrigel invasion assay. A Transwell chamber system (24 wells; 8 mm pore size; BD Biosciences, Franklin Lakes, NJ, USA) was used in A549 and NCI-H520 cell lines. The Transwell filters (polycarbonate membrane) were pre-coated with Matrigel (cat. no. 356234; BD Biosciences). Tumor cells were pre-incubated with or without 50 µM PD98059 (cat. no. 9900; Cell Signaling Technology, Inc.) at 37˚C for 1 h, following which 2x10^5 cells suspended in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 ng/ml CCL21 (cat. no. 300-35; PeproTech) were loaded on Matrigel in the upper chamber. RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was added to the lower chamber. Following 48 h incubation at 37˚C, noninvasive cells on the upper surface of Matrigel membrane (cat. no. 356234; BD Biosciences) were removed with a cotton swab. The invading cells on the underside of the Matrigel membrane (cat. no. 356234; BD Biosciences) were fixed with methanol for 30 min at 37˚C, stained with 1% crystal violet for 30 min at 37˚C and counted in at least five random separate fields under a light microscope. The assay was performed in triplicate.

Immunohistochemistry. Lung adenocarcinoma tissues were selected from 50 patients (29 females and 21 males) who underwent radical resection of lung cancer and had been clinically and histopathologically diagnosed in Shandong Tumor Hospital (Jinan, China). The tissues were blocked by 10% formalin and embedded in paraffin. No patients had received chemotherapy or radiation therapy prior to surgery. The TNM stage system (19) was applied to classify the specimens. Clinical pathological information was obtained from patient records in hospital and is summarized in Table I. All samples used in the study were anonymized and informed consent was received from all patients. The procedure in the present study was approved by the Medical Ethical Committee of Shandong University (Jinan, China).

The paraffin-embedded tissues were cut into ~5 µm-thick sections, and immunohistochemical staining was performed according to the manufacturer’s protocol. Tissue sections were de-waxed in xylene and rehydrated in a graded alcohol series. Antigen retrieval was conducted. The endogenous peroxidase of tissues was inactivated afterwards. Tissue slides were incubated with the following primary antibodies at 4˚C overnight: anti-Vimentin (dilution, 1:200; cat. no. 5741P; Cell Signaling Technology, Inc.), anti-Slug (dilution, 1:200; cat. no. sc-166476; Santa Cruz Biotechnology, Inc.), anti-CCL21 (dilution, 1:200; cat. no. sc-5808; Santa Cruz Biotechnology, Inc.) and anti-CCR7 (dilution, 1:500; cat. no. ab32527; Abcam). PBS was used as negative control. Using the EnVision System (Dako; Agilent Technologies, Inc.) primary antibodies were visualized, and nuclei were counterstained by hematoxylin. Immunostaining scoring for staining intensity and positive percentage in five high-power fields was performed according to a previous protocol under a light microscope (magnification, x400) (11). The intensity score was obtained by background staining as was graded as follows: None, 0; mild, 1; moderate, 2; intense, 3. The percentage of positive tumor cells were assessed according to the following patterns: 0% cells, 0; 0-25% cells, 1; 25-50% cells, 2; >50% cells, 3. The final score was obtained by multiplying the positive percentage score by the intensity score for each section, which ranged from 0 to 9. If the final score was <4, the tissue sections were classified as negative or low expression. In

| Parameter               | CCR7     | Slug     | Vimentin |
|-------------------------|----------|----------|----------|
|                         | Low      | High     | P         | Low    | High    | P         | Low    | High    | P         |
| Age                     | 0.887    | 0.623    | 0.598    |
| ≤50                     | 26       | 5        | 21       | 5      | 21      | 10        | 16     |
| >50                     | 24       | 5        | 19       | 6      | 18      | 11        | 13     |
| Sex                     | 0.254    | 0.793    | 0.917    |
| Female                  | 29       | 4        | 24       | 6      | 23      | 12        | 17     |
| Male                    | 21       | 6        | 16       | 5      | 16      | 9         | 12     |
| Lymph node status       | 0.010a   | 0.030a   | 0.030a   |
| Positive                | 28       | 2        | 26       | 3      | 25      | 8         | 20     |
| Negative                | 22       | 8        | 14       | 8      | 14      | 13        | 9      |
| Stage                   | 0.063    | 0.055    | 0.235    |
| I                       | 9        | 2        | 7        | 3      | 6       | 5         | 4      |
| II                      | 4        | 6        | 8        | 6      | 8       | 9         | 5      |
| III                     | 12       | 1        | 11       | 1      | 11      | 4         | 8      |
| IV                      | 15       | 1        | 14       | 1      | 14      | 3         | 12     |

*P<0.05 vs. lymph node status. CCR7, C-C chemokine receptor type 7.
contrast, if the score ≥4, they were regarded as positive or high expression.

The same method was applied to assess the expression of CCR7, Vimentin and Slug in interstitial stromal cells.

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. A chi-squared test was used for evaluating the association between CCR7 and EMT biomarkers in human lung carcinoma and lymphoid tissues. Differences among groups were compared by one-way analysis of the variance and a Tukey post hoc test. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was carried out using SPSS software (version, 18.0; SPSS Inc., Chicago, IL, USA).

Results

Differential expression of CCR7 and CCL21 in human lung cancer cells. To detect CCR7 and CCL21 expression in different lung cancer cell lines, western blot analysis was performed. CCR7 was expressed at different levels in H157, A549, 973 and H520 cell lines, appearing visibly increased in H520 and A549 cells (Fig. 1A); therefore, H520 and A549 cell lines were selected for the following assays. However, CCL21 was not detected in all cell lines (data not shown).

EMT phenotype expression is induced by CCL21 stimulation in lung cancer cells. To evaluate the roles of CCL21/CCR7 in activating EMT processes in human lung cancer cells, H520 and A549 cells were treated with or without 100 ng/ml CCL21 for 48 h. The results of the western blot analysis demonstrated that CCL21 may promote EMT biomarker protein expression in lung cancer cells, including decrease of E-cadherin and increase of Vimentin and Slug (Fig. 1B).

ERK signaling is activated in response to CCL21 stimulation in lung cancer cells. To investigate whether the ERK signaling pathway may be activated by CCL21, H520 and A549 cells were treated with or without CCL21 in different concentrations (0, 50, 100 or 200 ng/ml) for 48 h, and the protein expression levels of p-ERK were detected by western blot analysis. The results demonstrated that ERK phosphorylation was positively increased in response to CCL21 in a dose-dependent manner (Fig. 1C).

Inhibition of ERK phosphorylation blocks EMT transformation in response to CCL21/CCR7 in lung cancer cells. To investigate the potential roles of ERK1/2 in CCL21/CCR7-mediated EMT activation in human lung cancer cells, PD98059, a selective inhibitor of MEK, was used to disrupt activation of its downstream target, ERK. H520 and A549 cells were pre-treated with or without PD98059 in different concentrations (0, 50, 100 or 200 ng/ml) for 48 h, and the protein expression levels of p-ERK were detected by western blot analysis. The results demonstrated that ERK phosphorylation was positively increased in response to CCL21 in a dose-dependent manner (Fig. 1C).

ERK1/2 promotes migration of human lung cancer cells in response to CCL21/CCR7. The influence of ERK1/2 on cancer cell migration behavior was investigated using a wound-healing assay. H520 (Fig. 3A) and A549 (Fig. 3B) cells were cultured with or without PD98059, an inhibitor of MEK, followed by 100 ng/ml CCL21 stimulation for 48 h. The results revealed that there were more cancer cells migrating to the wound area in response to CCL21 stimulation, whereas this was markedly inhibited when the cells were pre-treated with PD98059 (P<0.01; Fig. 3C).

ERK1/2 increases invasion of human lung cancer cells in response to CCL21/CCR7. The influence of ERK1/2 on invasiveness of human lung cancer cells was tested using a Matrigel invasion assay. Following
pretreatment with PD98059 in H520 and A549 cells, followed by 100 ng/ml CCL21 stimulation for 48 h, the invaded cells travelling through the Matrigel were counted. The results indicated that the number of invasive cells in H520 (Fig. 4A) and A549 (Fig. 4B) samples were increased in response to CCL21 stimulation, whereas this was inhibited remarkably.

Figure 2. Roles of CCL21/CCR7 and p-ERK in EMT of lung cancer cells. (A) Western blot and (B) reverse transcription-quantitative polymerase chain reaction. Cancer cells presented a downregulation of E-cadherin and upregulation of Vimentin, Slug and p-ERK in response to CCL21 stimulation, when compared with the control group. These phenotype transformations in response to CCL21 stimulation above were abolished when the cells were pretreated with PD98059. Data are presented as the mean ± standard deviation of three independent experiments. "P<0.01. CCL21, chemokine (C-C motif) ligand 21; CCR7, C-C chemokine receptor type 7; ERK, extracellular-regulated signal kinase; EMT, epithelial-mesenchymal transition.
when the cells were pretreated with PD98059 (P<0.01; Fig. 4C).

**CCR7 expression correlates with clinicopathological parameters and Vimentin/Slug expression in human lung cancer tissues.** Immunohistochemical staining demonstrated that CCR7 and Vimentin were expressed in the cytoplasm and/or plasma membranes of tumor cells, and Slug was expressed mainly in the nucleus and partially in the cytoplasm of cancer cells (Fig. 5). Furthermore, CCR7, Vimentin and
Slug were additionally detected in 6 cases of interstitial stromal cells in cancer regions and/or in non-neoplastic tissue adjacent to carcinoma (data not shown), and the expression in interstitial stromal cells was assessed as above. The final score consists of tumor and interstitial stromal cells. CCR7, Slug and Vimentin were all highly expressed in lung carcinoma tissues with positive rates of 80% (40/50), 78% (39/50) and 58% (29/50), respectively. Associations between expressions of CCR7/Slug/Vimentin and clinical parameters were analyzed and are presented in Table I. The expression levels of CCR7, Slug and Vimentin were significantly associated with lymph node metastasis (P=0.010, P=0.030 and P=0.030, respectively). Additionally, CCR7 expression was correlated positively with expressions of Slug (R=0.459, P=0.001) and Vimentin (R=0.385, P=0.006). However, CCL21, the CCR7 ligand, was expressed mainly in the endothelium of lymphatic vessels adjacent to cancer cells (Fig. 5), but only weakly positive in cytoplasm of cancer cells in 34% (17/50) cases, which includes 20% (10/50) cases in the lymph node non-metastasis group and 14% (7/50) cases in the lymph node non-metastasis group. No significant differences were observed between these two groups.

**Discussion**

In the present study, the expression levels of CCR7 and CCL21 in various human lung cancer cell lines was analyzed. The results indicated that CCR7 was expressed in different cancer cell lines, and was most highly expressed in H520, a lung squamous cancer cell line, and A549, a lung non-small cell cancer cell line. However, CCL21 was not detected in any cell lines in vitro.

Based on the detection above, the expression levels of CCR7 and CCL21 in human lung cancer tissues were assessed by immunohistochemical staining. Similar to the findings in cancer cell lines, CCR7 was highly expressed in cancer cells in 80% of cancer tissues, and was associated with lymph node metastasis. Nevertheless, CCL21 was primarily expressed in the endothelium of lymphatic vessels surrounding the cancer foci, but hardly in cancer cells. Therefore, CCL21, released by

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**Figure 4. Evaluation of invasive capacity in human lung cancer cells following ERK pathway inhibition.** (A) H520 and (B) A549 cells were subjected to a Matrigel invasive assay. (C) The number of invasive cells treated with CCL21 was increased significantly in H520 and A549 cells, whereas this was significantly inhibited when the cells were pretreated with PD98059. Cells were imaged under a phase-contrast microscope. Magnification, x200. Scale bars=50 µm. **P<0.01. ERK, extracellular-regulated signal kinase; CCL21, chemokine (C-C motif) ligand 21.
lymphatic endothelium, may facilitate cancer cell migration to the lymphatic vessels. H520 and A549 cells were selected to evaluate whether CCL21/CCR7 activated EMT and the ERK signaling pathway in vitro.

The results demonstrated that CCL21/CCR7 effectively induced EMT via the ERK signaling pathway in the H520 and A549 lung cancer cell lines. Following treating with CCL21, the EMT phenotype was accelerated in H520 and A549 cells. The epithelial marker E-cadherin was downregulated, whereas the mesenchymal markers, Vimentin and Slug, were upregulated. Immunohistochemical detection revealed that expression of CCR7 was positively associated with EMT Vimentin and Slug expression in human lung carcinoma tissues, consistent with the in vitro findings. Additionally, these results were consistent with previous studies in breast and gastric cancer cells (15,16). EMT causes epithelial cells to acquire invasion and migration capacities (1,2). Therefore, the CCL21/CCR7 axis not only determines the targeted migration, but also promotes cancer cell migration.

The effect of the ERK signaling pathway on CCL21/CCR7 was investigated to identify whether it activates EMT in human lung cancer cells in vitro. Following inhibition of ERK phosphorylation, invasion and migration of cancer cells were significantly decreased compared with cells without ERK inhibition. In addition, the mRNA and protein expression levels of EMT biomarkers were reduced in response to CCL21 stimulation. These results indicated that CCL21/CCR7 activates the EMT procedure by activating ERK phosphorylation. ERK1/2 belongs to the mitogen-activated protein kinase family, working as the most common signaling pathway in mediating EMT (20,21). Therefore, ERK is a critical signal concerned with chemotaxis and migration of cancer cells.

It has been demonstrated by immunohistochemical staining that CCR7 is expressed in a variety of carcinomas (4,22); however, its ligands CCL19 or CCL21 have rarely been investigated in previous reports. A report previously demonstrated that a melanoma cell line and several breast cancer cell lines secreted the CCR7 ligands, CCL21/19 (23), which may promote the migrating capacity of cancer cells with high levels of CCR7. However, in the present study, CCL21 was expressed at low levels in lung cancer cells, although it was expressed strongly in the endothelium of lymphatic vessels. Lymphatic vessels often exist around the cancer foci, so certain cancer cells are situated far from lymphatic vessels. Therefore, the present study aimed to identify which factor may stimulate CCR7 expression when cancer cells do not secrete CCR7 ligands. A previous study identified that, under hypoxic conditions, hypoxia-inducible factor (HIF)-1α and HIF-2α upregulate CCR7 expression in non-small lung cancer via the ERK1/2 signaling pathway (24,25). An additional study confirmed that TGFβ-1 may stimulate cancer cells to express CCR7 (26). TGFβ-1, a typical cytokine for EMT stimulation, may be released widely by various cells including cancer cells in the tumor microenvironment (27). Therefore, these factors (except CCR7 ligands) may serve as more significant stimulators for CCR7 expression of cancer cells in the cancer microenvironment. This allows cancer cells rich in CCR7 to easily initiate EMT and chemotaxis processes in response to CCL21/19 released by the capillary lymphatic endothelium.

In conclusion, CCL21/CCR7 may trigger EMT via the ERK1/2 signaling pathway in human lung cancer cells. The present study provided evidence that a close interaction exists between EMT and CCL21/CCR7 chemotaxis processes in lung cancer metastasis. Targeting the signal that connects CCR7 and EMT may become an important strategy for oncotherapy. However, additional research is required regarding the network and molecular interaction of signal transduction pathways.

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