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Original article

Chemokine receptor 9 high-expression involved in the migration and invasion of the non-small-cell lung cancer cells

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Background: Metastasis is responsible for most cancer-related death, and the metastatic spread of neoplastic cells may be related to the ability of migration and invasion. Chemokine receptor 9 (CCR9) plays an important role in cutaneous melanoma and prostate cancer cells migration and invasion.
Objective: Investigate the specific role of the chemokine-ligand (CCR9-CCL25) axis in the development of nonsmall cell lung cancer (NSCLC) metastasis.
Methods: Semi-quantitative reverse transcriptase-PCR, western-blot, flow cytometry, migration, and invasion assays were used to examine the function of CCR9 in the NSCLC cells.
Results: CCR9 was highly expressed in NSCLC patient cancer tissue. In addition, in vitro migration and invasion studies on human bronchial epithelial cells of the BEAS-2B and human squamous lung cancer cell lines NCI-H157 showed that migration in response to the CCL25 was inhibited by CCR9 antibody.
Conclusion: CCR9 might play an important role in the migration and invasion of the NSCLC cells.

Keywords: CCR9, CCL25, non-small cell lung cancer, migration, invasion

Lung cancer is the leading cause of cancer death in both men and women. Non-small cell lung cancer (NSCLC) represents 85% lung cancer cases [1]. In almost two-thirds of cases, the cancer has already spread beyond localized disease at the time of diagnosis, limiting therapeutic options [2, 3]. Therefore, it is important to investigate the mechanisms of the migration and invasion of the NSCLC cells.

Chemokine receptor 9 (CCR9) is a member of G protein-coupled receptor (GPCR), and plays an important role in T-cell development and tissue-specific homing after binding with its specific ligand, CCL25 [4-6]. CCR9 is expressed on human melanoma cells [7, 8], breast cancer cells [9], and prostatic carcinoma cells [10]. It participates in the enhanced motility of these cancer cells, being likely a "homing receptor" for many cancers to migrate and invade. Many reports suggest that the chemokine-ligand axis (CCR9-CCL25 axis) is involved in organ-specific trafficking of tumor metastasis. CCL25 may be the predominant chemokine involved in cellular migration of melanoma, ovarian cancer, breast cancer, leukemia, and prostatic carcinoma in select cells or in specific clinical situations [10-14].

In this study, we investigated the specific role of the CCR9-CCL25 axis in the development of NSCLC metastasis. Using semi-quantitative reverse transcriptase-PCR, western-blot, flow cytometry, migration and invasion assays, we examined the function of CCR9 in the NSCLC cells, and the correlation between CCR9 expression and the enhanced migration and invasion of NSCLC cells when induced by the CCR9 ligand-CCL25.

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Materials and methods

Patients and tissue

Fifty primary NSCLC patients treated at the People's Hospital, Wuhan University between 2008 and 2009 were enrolled in our study, including 32 males and 18 females with a median age of 51 years. All of the patients gave informed consent according to institutional guidelines. The study was approved by the Ethical Review Board for Research in Wuhan University.

Lung tumors were categorized in terms of histological type, grade, and stage according to WHO standards [15]. The international tumor-node-meta-stasis (TNM) staging in patients are summarized in **Table 1**. The cancer tissues and paired normal tumor-adjacent tissues were fresh and received from the People's Hospital, Wuhan University. They were frozen in liquid nitrogen until process for future examination. Patients were not pre-treated with radiotherapy or chemotherapy prior to surgery. All specimens were confirmed by pathological examination.

Cells culture

Human bronchial epithelial cells of the BEAS-2B cell line (ATCC No. CRL-9609) [16] were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) with 10% heat-inactivated fetal bovine serum, penicillin 100 IU/mL, and streptomycin 10 μg/mL. Cells were grown and maintained in cell culture flasks at 37°C in a 5% CO_2 humidified incubator. $HgCl_2$ solution was prepared in phosphate-buffered saline (PBS) and diluted with the same culture medium using 10% fetal bovine serum when applied to the cells. NCI-H157 cells (ATCC No. CRL-5802) [17] were derived from a human squamous lung cancer. They were cultured in RPMI medium (Life Technologies Inc. Carlsbad, USA) with 10% enriched calf serum (Gemini Bioproducts, Woodland, USA).

Extraction of total RNA and semi-quantitative reverse transcriptase (RT) -PCR

Total RNA was extracted from tissues and cultured cell lines with TRIzol (Invitrogen, Carlsbad, USA) according to the user manual. cDNA was prepared

Table 1. Characteristics in patients with NSCLC based on the international tumor-node-meta-stasis (TNM) staging.

	Histology	Number of patients
	AC	33
	SCC	17
T		
	T1	12
	T2	30
	T3	6
	T4	2
N		
	N0	31
	N1	9
	N2	7
	N3	3
M		
	M0	39
	M1	11
Stage		
	I	25
	II	7
	III	8
	IV	10

AC=adenocarcinoma, SCC=squamous cell carcinoma. The TNM Classification of Malignant Tumors (TNM) is a cancer staging system that describes the extent of cancer in a patient's body. T describes the size of the tumor and whether it has invaded nearby tissue. N describes regional lymph nodes that are involved. M describes distant metastasis.

from total RNA by using a First Strand cDNA Synthesis kit (Roche, Indianapolis, USA). CCR9 mRNA expressions were detected in 50 NSCLC patients cancer tissues, paired normal tumor-adjacent tissues and cultured cell lines by quantitative RT-PCR analysis. First-strand cDNA template was synthesized using Super-Script II reverse transcriptase (Life Technologies, Carlsbad, USA) with random hexamers. Serial dilutions of the secDNA templates were subjected to PCR amplification using sets of primers of CCR9 (forward primer, 5-ATTGCACAA GAGTGAAGACC-3; reverse primer, 5-GTCAAC AGCCTGCACTACAA-3) or GAPDH (forward primer, 5-CCCCACACACATGCACTTACC-3; reverse primer, 5-CCTACTCCCAGG GCTTTGAT T-3). Cycling parameters were 30 seconds at 94°C, 45 seconds at 56°C, and one min at 72°C for 35 cycles to detect CCR9 mRNA, and 50°C for two minutes, then 95°C for 10 minutes, followed by 40 cycles of 60°C, one minute and 95°C, 15 seconds for GAPDH. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide.

Western-blot analysis

Dissect the NSCLC cancer tissues and normal tumor-adjacent tissues were dissected on ice and as quickly as possible to prevent degradation by proteases. The tissues were placed in Eppendorf tubes and kept on ice for immediate homogenization with tissue lyses buffer according to the manufacture instructions. The tubes were gently removed from the centrifuge and placed on ice. Then, the supernatant was aspirated and placed in a fresh tube kept on ice. After concentration measured by the Bicinchoninic acid (BCA) method, equal amounts of protein were electrophoresed on 12% SDS/polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membranes (PVDF) (Millipore, Billerica, USA) by electroblotting. After blocking for one hour in Tris-buffered saline (pH 7.6, containing 0.1% Tween and 5% non-fat milk) at room temperature, membranes were incubated overnight at 4°C with primary anti-CCR9 monoclonal antibody (R&D Systems, Minneapolis, USA) at 1:1000 dilution, and anti-GAPDH antibody (Abcam, Cambridge, UK) at 1:2000 dilution with gentle shaking. After washing, the membrane was then probed with the appropriate secondary antibody for 60 minutes at room temperature. Protein binding on the membrane was detected by the enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, USA) according to the manufacturer's instructions. Then, band intensity was measured using densitometry with the Quantity One software (Bio-Rad, Hercules, USA). The protein levels were normalized with respect to β-actin protein. The method for the analysis of the BEAS-2B and NCI-H157 cells is the same as above.

Flow cytometry analysis

Phycoerythrin (PE)-conjugated mouse anti-human CCR9 antibody was purchased from R&D Systems (Minneapolis, USA). PE-conjugated mouse IgG2a monoclonal immunoglobulin isotype control was purchased from PharMingen (San Diego, USA). NCI-H157 cells and BEAS-2B cells were first incubated with PE-labeled anti-human CCR9 antibody or matched isotype antibody at 5 μg/mL in PBS containing 2% Bovine serum albumin (BSA) and 0.1% sodium azide for 20 minutes, followed by washing twice in staining buffer. The analyses were performed with a flow cytometer.

Migration and invasion assays

CCL25 was obtained from PeproTech. Unlabeled human CCR9 mAb (clone 112509) was purchased from R&D Systems. Migration study was performed using Matrigel invasion chamber (Becton Dickinson Labware, Bedford, USA). RPMI 1640 was added to the interior of the bottom and top chamber of inserts and allowed to hydrate for two hours at 37°C with 5% CO_2. CCL25 or albumin at 100 ng/mL concentration was added to the bottom chamber. Next, 10^4 cells were added to the top chamber of inserts. To block chemokine receptor interaction, 1.0 μg/mL mouse antihuman CCR9 was added to the top chamber of Matrigel or control inserts and incubated overnight at 37°C and 5% CO_2. After incubation, cells at the bottom surface of the insert were fixed with 100% methanol for two minutes, stained for two minutes in 1% toluidine blue (Sigma) supplemented with 1% borax (Sigma), and rinsed twice with distilled H_2O. Cells were counted under a microscopy at x 40 fields [10, 18, 19].

For invasion study, the Matrigel diluted to 1 mg/mL with serum free medium was used to coat the upper side of the Transwell membranes, the identical assay and staining were same as the migration study, except the lower side of the Transwell membrane was also pre-coated with 20 μg/mL Matrigel. After

incubated for one hour at 37°C, replaced with the conditioned medium and then the cells were incubated overnight before fixation [10, 18, 19].

Statistical analysis

Statistical analysis was performed using Mann-Whitney's U test and the Kruskal-Wallis test. Values are expressed as the mean±SD. A 95% confidence limit was considered to be significant ($p < 0.05$).

Results

Expression analysis of CCR9 in NSCLC tissues by semi-quantitative RT-PCR and Western blot.

Figure 1 shows that the level of CCR9 mRNA was highly expressed in NSCLC tissues by semi-quantitative RT-PCR ($p < 0.05$).

Western-blot result showed that the protein level of CCR9 was significantly up-regulated in lung cancer tissues compared with the paired normal tumor-adjacent tissues.

CCR9 expression analysis in BEAS-2B and NCI-H157 cell lines

Figure 3 shows that CCR9 was highly expressed in human squamous lung cancer cell line NCI-H157 *vs.* control cells.

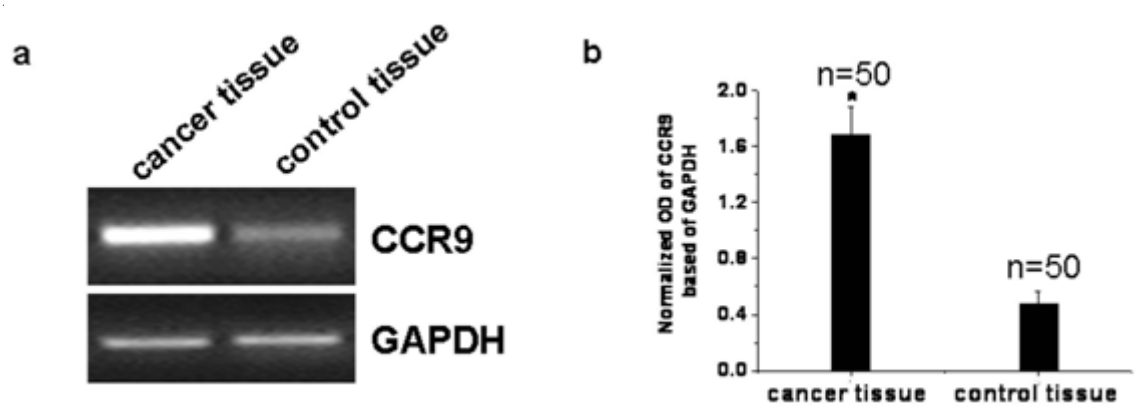

Fig. 1 CCR9 mRNA expressed in NSLC and normal tumor-adjacent tissues (**a**), and the CCR9 mRNA expressed relative to copies of GAPDH (**b**). CCR9 was highly expressed in NSLC tissues compared with the paired normal tumor-adjacent tissues (*$p < 0.05$).

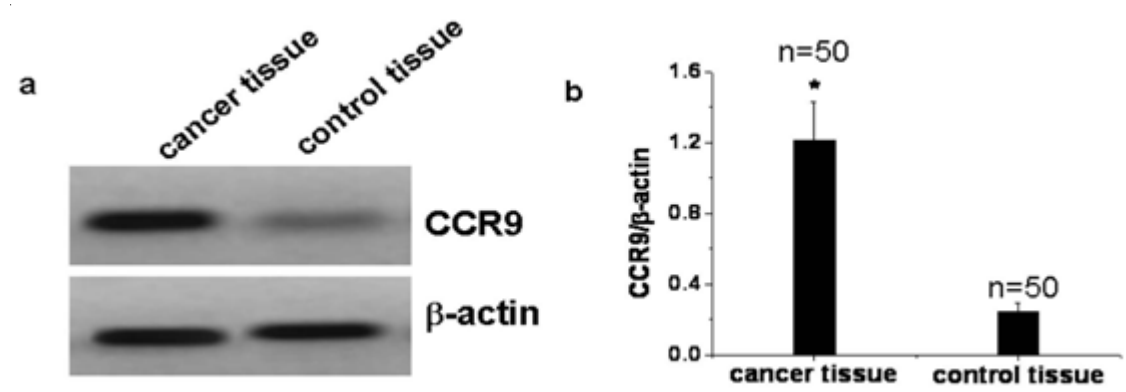

Fig. 2 **a**: Expression analysis of CCR9 by Western-blot. Cancer tissues and control tissues were collected, treated, and blotted as described in the Methods. **b:** The expression of CCR9 relative to actin in NSLC tissues is significantly higher than the paired control tissues (*$p < 0.05$)

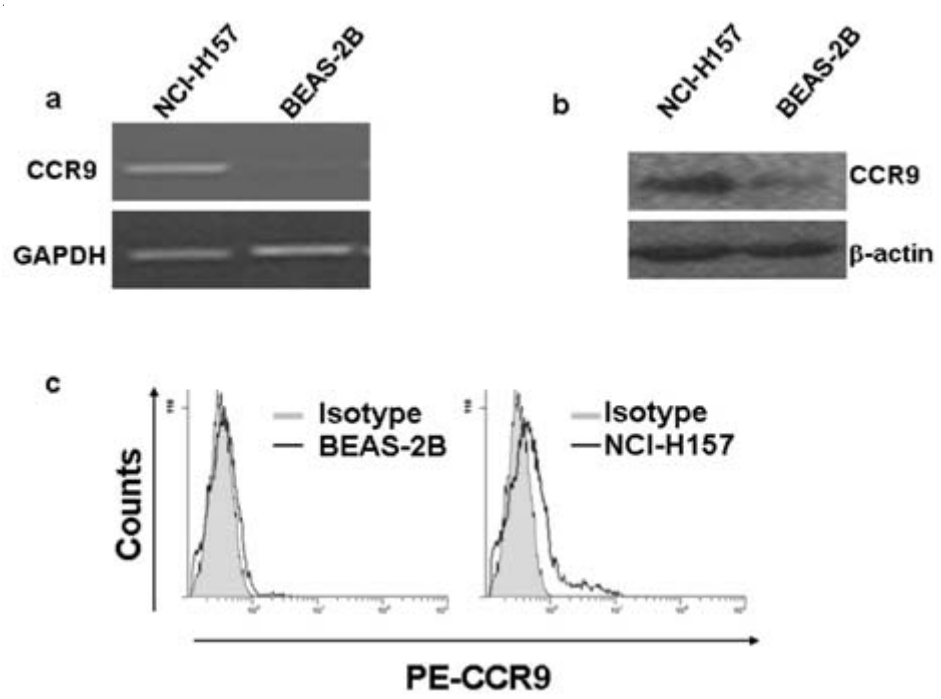

Fig. 3 CCR9 expression analysis on cell lines. Human squamous lung cancer cell line NCI-H157 and human bronchial epithelial cell line were cultured, and the CCR9 expression was analyzed with semi-quantitative RT-PCR, western blot and FACS. The positive CCR9 surface expression by these two cells was repeated for three times.

CCR9 promote the migration and invasion of the NCI-H157 cells

Figure 4 shows that the number of NCI-H157 cells that migrated in response to CCL25 was significantly higher than that of control cell BEAS-2B.

In addition, the proportion of NCI-H157 cells that were invasive after CCL25 induced was also increased. Moreover, NCI-H157 cells that expressed higher level of CCR9 were more invasive than the BEAS-2B cells. The number of migration and invasion is almost 0 in BEAS-2B cells (**Fig. 5**).

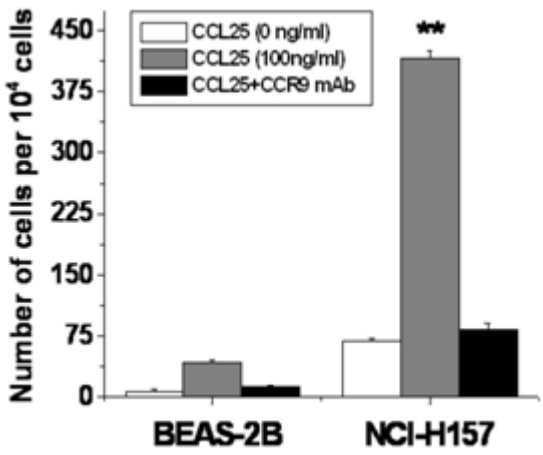

Fig. 4 CCR9-mediated NSLC migration. Human squamous lung cancer cell NCI-H157 and human bronchial epithelial cell were tested for their ability to migrate to 0 ng/mL CCL25, 100 g/mL anti-CCR9 monoclonal antibody in the 0 ng/mL CCL2, or 100 ng/mL of CCL25+1 lower chamber. The number of NCI-H157 cells which migrated in response to CCL25 was significantly higher than that of control cell BEAS-2B (**$p<0.01$). The migration can be blocked by anti-CCR9 antibody.

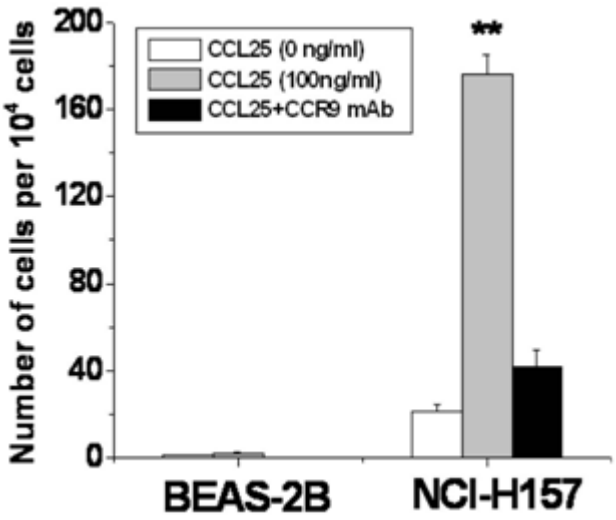

Fig. 5 CCR9-mediated NSLC cell invasion. Two cells were tested for their ability to invade across a Matrigel matrix in response to chemotactic gradients of 0 ng/mL CCL25, 100 ng/mL CCL25, g/mL anti-CCR9 monoclonal antibody in the lower chamber, or 100 ng/mL CCL25 + 1, consistent with the migration results. The number of NCI-H157 cells that invaded in response to CCL25 was significantly higher than that of control cell BEAS-2B (**p <0.01). The invasion can be blocked by anti-CCR9 antibody.

Discussion

Most NSCLC-related deaths are not a result of primary tumor growth, but are rather caused by the spread of cancer to other organs [20]. Many molecular factors may contribute to the metastasis of lung cancer cells. The chemokines are involved in the migration and invasion of various tumor cells, causing cancer cells to "home" to specific secondary sites to promote organ-specific metastasis and chemokine receptors. Their corresponding chemokine ligands have been demonstrated to play an important role in cancer metastasis. Previous studies have showed that chemokine receptors may promote tumor diffusion at several key steps of metastasis, including adherence of tumor cells to endothelium, metastatic colonization, etc. [21-23]. Some studies showed that the CCR9-CCL25 interaction might be the critical targets for the carcinoma cell's migration and invasion to specific and rare organs [24]. For example, CCR9 was detected in some carcinoma cells and mediated their organ-selective metastasis. Singh et al. [10] found that the prostate cancer cell lines PC3 and LNCaP were highly expressed CCR9, while the LNCaP cells that possessed higher CCR9 expression were more invasive than PC3 cells after CCL25 induction. Johnson et al. [25] found that CCL25-CCR9 interaction modulates ovarian cancer cell migration, metalloproteinase expression, and invasion. Sharma et al. [26] showed that CCR9 mediates PI3K/AKT-dependent antiapoptotic signals in prostate cancer cells and inhibition of CCR9-CCL25 axis could enhances the cytotoxic effects of etoposide.

We hypothesized that chemokines mediate the migration and invasion of the NSCLC cells. To address our hypothesis, we first detected the expression of chemokine receptors by both primary NSCLC tissues and the human squamous lung cancer cell line NCI-H157. Although these receptors were expressed in normal tumor-adjacent tissue, the level of their expression was significantly higher in NSCLC tissues compared with the paired normal tumor-adjacent tissues. These findings suggested that CCR9-CCL25 axis could mediate the migration and invasion of the NSCLC cells. Subsequently, we tested the expression of CCR9 in the human squamous lung cancer cell line NCI-H157 in order to identify further the relationship between CCR9 and NSCLC. The data prompted that the expression level of CCR9 was higher than that of BEAS-2B cells, which is a cell model of human bronchial epithelial cells. Moreover, the biological and functional significance of CCR9 expression was also tested by migration and invasion studies in vitro. The results also revealed that CCL25 could increase the migration and invasion of CCR9-expressing NSCLC cell line NCI-H157. Interestingly, when CCR9 specific antibody was used in the

migration and invasion studies, we could find that it markedly reduced the migration and invasive potential of the NCI-H157 cells. This indicates that the migration and invasion of NSCLC might depend on the CCR9 and its receptor CCL25.

Recently, it has been shown that chemokine-receptor including CXCR4, CCR9 is expressed by various cancer cells, and correlated with the migration and invasion of these tumors. Additionally, anti-CXCR4 antibodies and anti-CCR9 antibodies block the metastatic spread of breast tumors to the lungs and the migration and invasion of prostate cancer cell, respectively [10, 27]. Although it has been shown that CCR9 is expressed in the thymus, lymph nodes and spleen, and T cells [28, 29], very little is known about the CCR9 by the NSCLC.

In our study, CCR9 was expressed by NSCLC tissues and cell line. Moreover, CCR9 expression played a role in migration and invasion of the NSCLC. We hypothesized that NSCLC cells with the ability of metastasis might express CCR9. This possibility of such a CCR9-dependent event was demonstrated by the inability of anti-CCR9 antibody-treated cells to migrate or invade. The expression of CCR9 by NSCLC suggested that CCR9-CCL25 interactions might play a significant role in NSCLC metastasis. Expression of functional CCR9 by prostate cancer cells, together with selective ligand expression by bone marrow and lymph nodes, supports our hypothesis that the migration and invasion of metastatic cancer cells may be chemokine-mediated. However, it will be necessary to clarify further the precise mechanisms of chemokine-mediated migration and invasion of NSCLC cells.

In conclusion, CCR9 might play an important role in the migration and invasion of the NSCLC cells.

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