

CCL25 promotes the migration and invasion of non-small cell lung cancer cells by regulating VEGF and MMPs in a CCR9-dependent manner

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Abstract. The CC chemokine receptor 9 (CCR9) and its natural secreted ligand CC motif chemokine ligand 25 (CCL25) have been implicated in cancer metastasis. However, their metastatic potential in non-small cell lung cancer (NSCLC) remains unclear. In the present study, immunohistochemistry was used to detect the expression and localization of CCR9, vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-1 and MMP-7 in lung cancer tissue and adjacent normal tissue. The association between the expression of CCR9 and clinical variables was also examined. Reverse transcription-quantitative PCR and western blotting were conducted to detect the expression of VEGF-C, VEGF-D, MMP-1 and MMP-7 in lung cancer cell lines (A549 and SK-MES-1). Migration and invasion assays were conducted to examine cell migration and invasion. Survival and mutation analysis were conducted using published datasets. The expressions of CCR9,

VEGF, MMP-1 and MMP-7 were upregulated in cancer tissue, compared with adjacent normal tissue (all $P < 0.05$). Patients with lower expression of CCR9 or CCL25 had better overall survival (OS) compared with those with higher CCR9 or CCL25 expression ($P < 0.05$ and $P = 0.05$, respectively). Furthermore, the expressions of VEGF-C, VEGF-D, MMP-1 and MMP-7 were higher in the CCL25-treated cell lines (all $P < 0.05$), but MMP-7 protein expression was not affected by CCL25 treatment in SK-MES-1 cells ($P > 0.05$). Following treatment with CCL25, lung cancer cells demonstrated higher migratory and invasive potential, which could be blocked by the CCR9 antibody ($P < 0.05$). Survival analysis demonstrated that low expression levels of both CCR9 and CCL25 mRNA indicated favorable OS in patients with NSCLC. Altogether, these results suggested that CCL25 enhanced the phenotype associated with migration and invasion in NSCLC by regulating the expression of VEGF-C, VEGF-D, MMP-1 and MMP-7.

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Abbreviations: NSCLC, non-small cell lung cancer; CCR9, CC chemokine receptor 9; CCL25, CC motif chemokine ligand 25; VEGF-C, vascular endothelial growth factor-C; VEGF-D, vascular endothelial growth factor-D; MMP-1, matrix metalloproteinase-1; MMP-7, matrix metalloproteinase-7; SCC, squamous cell carcinoma; AC, adenocarcinoma; OS, overall survival

Key words: non-small cell lung cancer, chemokine receptor 9, CC motif chemokine ligand 25, metastasis

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, 85% of which is non-small cell lung cancer (NSCLC) (1), which usually invades the regional or distant lymph nodes, brain and liver (2,3). Metastasis is typically the most common risk of death in NSCLC, which suggests that blocking the migration and invasion of tumor cells might be a possible therapeutic strategy.

A previous study identified that migration and invasion of tumor cells could be regulated by chemokines and their receptors (4). Chemokines, which are a family of small, structurally related heparin-binding proteins classified as C, CXC, CC and CX3C sub-families (5,6), are essential to the normal and pathologic trafficking of leukocytes. The biological functions of chemokines are mediated via G-protein-coupled receptors, which play an important role in inflammation and leukocyte differentiation. It has been demonstrated that various cell types may express different chemokine receptors, such as C-X-C chemokine receptor type 2 (CXCR2) (7), C-X-C chemokine receptor type 4 (CXCR4) (8), C-C chemokine receptor type 6 (CCR6) (9) and C-C chemokine receptor type 9 (CCR9) (10).

High expression of CXCR2, CXCR4 and CCR9 with their respective ligands C-X-C motif chemokine 8, C-X-C motif chemokine 12 and CC motif chemokine ligand 25 (CCL25) was associated with tumor metastasis and poor survival in NSCLC (11-13). We previously demonstrated that CCR9 and CCL25 interaction could suppress apoptosis in a phosphoinositide 3-kinase/Akt-dependent model in the NSCLC cell lines A549 and SK-MES-1 (14). However, the exact molecular mechanism of migration and invasion in NSCLC remains elusive.

The present study aimed to examine the potential association between the CCL25/CCR9 axis and key molecules involved in lung cancer cell migration, and to verify further the association between the CCL25/CCR9 axis and key molecules examined (VEGF-C, VEGF-D, MMP-1 and MMP-7) by CCR9 drug treatment experiments. A Kaplan-Meier survival analysis was used to further confirm the association between the CCL25/CCR9 axis and prognosis of lung cancer.

Materials and methods

Study design. A total of 60 patients with NSCLC who underwent surgery in The Huadong Hospital Affiliated with Fudan University were enrolled in the present study between September 2012 and December 2015. Tumor tissues and adjacent normal tissues were collected and paraffin-embedded. Neither chemotherapy nor immunotherapy was performed prior to surgery. A double pathological diagnosis was performed prior to enrolling the patients in the present study. The median age of these patients was 58 years, with the age ranging from 31 to 76. Further patient information is presented in Table I. The present study protocol was approved by The Ethics Committee Review Board of The Huadong Hospital Affiliated with Fudan University, and written informed consent was obtained from every participant.

Immunohistochemistry (IHC) assay. Formalin-fixed (10%, for 8 h at room temperature), paraffin-embedded tissues were sliced into 4- μ m sections using a microtome, then successively deparaffinized with xylene, rehydrated in a descending alcohol series and rinsed with PBS followed by high-pressure antigen retrieval in a microwave oven with citrate buffer (100°C for 5 min and then 24°C for a further 3 min). Subsequently, sections were treated with 3% H₂O₂ for 10 min at room temperature to block endogenous peroxidase. The sections were washed with PBS three times for 10 min each and then blocked with 10% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature. The tissue sections were subsequently incubated with anti-CCR9 (1:200; cat. no. ab1662; Abcam), anti-vascular endothelial growth factor (VEGF; 1:200; cat. no. AF0312; Beyotime Institute of Biotechnology) anti-matrix metalloproteinase (MMP)-1 (1:500; cat. no. ab137332; Abcam) or anti-MMP-7 (1:500; cat. no. ab205525; Abcam) antibodies overnight at 4°C. The slides were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:50; cat. no. A0216, Beyotime Institute of Biotechnology) or HRP-conjugated goat-anti-rabbit antibody (1:50; cat. no. A0208; Beyotime Institute of Biotechnology) at room temperature for 60 min. Finally, the sections were washed with PBS, then

stained with diaminobenzidine and hematoxylin (Beyotime Institute of Biotechnology) at room temperature for 5 min. The sections were observed under a light microscope (magnification, x200; BX-43; Olympus Corporation). A semi-quantitative scoring system (15) was applied to assess the expression of CCR9, VEGF, MMP-1 and MMP-7 in the specimens based on the average intensity and density of positively stained cells by IHC. Staining intensity was graded as follows: i) 0, no staining; ii) 1, weak staining; iii) 2, moderate staining; and iv) 3, strong staining. The density of positively stained cells was scored according to the following criteria: i) 0, \leq 5%; ii) 1, 5-25%; iii) 2, 26-50%; and iv) 3, >50%. The total IHC score was calculated by multiplying the intensity score by the density score. A total IHC score >0 was defined as positive expression and a total IHC score=0 was defined as negative expression.

Cell culture. The NSCLC cell lines SK-MES-1 [squamous cell carcinoma (SCC)] and A549 [adenocarcinoma (AC)] were purchased from The Kunming Institute of Zoology, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gemini Bio Products), 100 U/ml penicillin and 100 μ g/ml streptomycin in 5% CO₂ at 37°C. Before migration and invasion studies, lung cancer cells were cultured for 24 h in RPMI-1640 with 2% FBS.

Reverse transcription-quantitative PCR (RT-qPCR) primer design. The human mRNA sequences for VEGF-C, VEGF-D, MMP-1, MMP-7 and GAPDH were obtained from The National Institutes of Health National Center for Biotechnology Information (Genbank Database Accession nos. NM_005429.4, NM_004469.4, NM_001145938.2, NM_002423.5 and BC004109, respectively). These sequences were used to design primers for RT-qPCR analysis. The primer sequences were as follows: VEGF-C forward, 5'-AGCACG AGCTACCTCAGCAAGAC-3' and reverse, 5'-TTTAGACAT GCATCGGCAGGAA-3'; VEGF-D forward, 5'-AGCTGC CTGATGTCAACTGCTTA-3' and reverse, 5'-GTTTCATTAC TGGAGCCCTGCAC-3'; MMP-1 forward 5'-AAGAATGAT GGGAGGCAAGT-3' and reverse, 5'-GGTTTCAGCATCTGG TTTCC-3'; MMP-7 forward 5'-GAGTGAGCTACAGTGGGA ACA-3' and reverse, 5'-CTATGACGCGGGAGTTTAACAT-3' and GAPDH forward, 5'-GACGGCAAGATGCACATCAC-3' and reverse, 5'-GAGATGTAGCACGGGATCATGG-3'.

RNA isolation and RT-qPCR analysis. Total RNA was isolated from lung cancer cells using the RNA Simple Total RNA kit (Tiangen Biotech Co., Ltd.). Cells were untreated, CCL25-treated or CCL25-treated and stained with anti-CCR9 antibody, cDNA was generated using the Quantscript RT kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocols. Real-time RT-qPCR was performed using a 2X Taq PCR MasterMix (Tiangen Biotech Co., Ltd.) in a ABI7500 instrument (Applied Biosystems; Thermo Fischer Scientific, Inc.) using the following thermocycling conditions: Initial denaturation for 3 min at 94°C, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, prior to final extension at 72°C for 5 min. The expression of GAPDH was measured in each sample as an endogenous control. The ratio of

mRNA expression was calculated for each group using the $2^{-\Delta\Delta Cq}$ method (16).

Western blot analysis. The A549 and SK-MES-1 cell lines were treated with 100 ng/ml CCL25 (cat. no. 300-45; PeproTech, Inc.) or CCL25 (100 ng/ml) with 5 μ g/ml anti-CCR9 antibody (cat. no. ab1662; Abcam) at 37°C and 5% CO₂ for 48 h. Untreated cells were used as controls. Total protein from untreated and treated cells was washed with cold PBS and isolated in modified radio-immunoprecipitation assay buffer (50 mM Tris-Cl at pH 7.5; 150 mM NaCl; 1% NP-40; 1.0% sodium deoxycholate; 1% PMSF and 0.1% SDS) and quantified using the bicinchoninic acid method (Beyotime Institute of Biotechnology). Proteins were loaded (100 μ g per lane) and resolved by SDS-PAGE on 10% gels using Tris-glycine-SDS buffer (25 mM Tris; 192 mM glycine and 0.1% SDS; pH 8.8). Proteins were transferred to polyvinylidene fluoride membranes (EMD Millipore) using a wet transfer apparatus (Bio-Rad Laboratories, Inc.). Membranes were incubated in blocking buffer (5% non-fat milk dissolved in TBS + 0.1% Tween-20) for 1 h at room temperature. After blocking, membranes were incubated with anti-VEGF-C (1:500; cat. no. ab83905; Abcam), anti-VEGF-D (1:800; cat. no. ab155288; Abcam), anti-MMP-1 (1:1,000; cat. no. ab137332; Abcam), anti-MMP-7 (1:1,000; cat. no. ab205525; Abcam) and anti-GAPDH (1:2,000; cat. no. ab8245; Abcam) antibodies overnight at 4°C. Membranes were then washed in wash buffer (TBS + 0.1% Tween-20) three times for 10 min each time. After washing, membranes were incubated with the secondary antibodies for 2 h at 37°C. The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit antibody (1:8,000; cat. no. ab205718; Abcam) or goat-anti-mouse antibody (1:8,000; cat. no. ab205719; Abcam). Following incubation, membranes were washed five times with washing buffer for 6 min each time. The immunoreactive bands were detected using the ECL Plus reagent (Beyotime Institute of Biotechnology). ImageJ software (version 1.5.2; <https://imagej.nih.gov/ij>) was used to quantify the data from three independent experiments. Bands from untreated cells were given a value of 1.0 and the protein levels of treated groups were normalized to this value.

Migration and invasion assays. Migration and invasion studies were performed using Corning Biocoat™ Matrigel® invasion chambers with 8- μ m pore size (Corning, Inc.). Serum-free RPMI-1640 was added to the bottom and top chambers and allowed to hydrate the membrane for 2 h at 37°C with 5% CO₂. Subsequently, 1×10^4 cells were seeded to the top chamber and 100 ng/ml CCL25 (PeproTech, Inc.) was added into the bottom chamber as a chemo-attractant. To determine if the migration and invasion of lung cancer cells was mediated specifically by CCL25/CCR9 interaction, cells pre-incubated with 5.0 μ g/ml anti-CCR9 antibody (Abcam) were added to the top chamber and allowed to migrate or invade in the presence of CCL25 at 37°C with 5% CO₂ overnight. After incubation, non-migrating cells in the upper surface of the membrane were removed. Cells at the bottom surface of the insert were fixed with 100% methanol for 10 min at room temperature, stained for 2 min with crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature and washed

twice with deionized water. Migrated or invaded cells were counted using a light microscope (magnification, x200). All experiments were repeated three times for validation.

Bioinformatics analysis. The Kaplan-Meier Plotter online tool (<http://kmplot.com/analysis/index.php?p=service&cancer=lung>) was used to investigate the association between CCR9 and CCL25 mRNA expression and clinical prognosis for patients with NSCLC (n=1,926) (17). In this database (<http://kmplot.com>), multiple independent transcriptomic datasets, generated using caBIG (<http://cabig.cancer.gov>), GEO (<http://www.ncbi.nlm.nih.gov/geo/>) and TCGA (<http://cancergenome.nih.gov>), are merged and the gene expression data and survival information were analysed, where the hazard ratio (HR), 95% confidence interval (CI) and log-rank P-value was determined and displayed. P<0.05 was considered to indicate a statistically significant difference. The mutational level of CCR9 and CCL25 was determined using the cBioPortal for Cancer Genomics website (<http://www.cbioportal.org/>).

Statistical analysis. All values are presented as the mean \pm SD. Associations between CCR9 expression and clinicopathological parameters were assessed using the χ^2 test as well as the Fisher's exact test. Statistically significant differences between groups were analyzed by one-way ANOVA with Tukey's post hoc test. Data were analyzed using SPSS 20.0 software (IBM Corp.) and GraphPad Prism 7 (GraphPad Software, Inc.). P<0.05 was used to indicate a statistically significant difference.

Results

CCR9, VEGF, MMP-1 and MMP-7 expression in NSCLC tissue. CCR9, VEGF, MMP-1 and MMP-7 were located on the cell membrane and/or the cytoplasm in the cancer tissues (Fig. 1). The positive rates of CCR9, VEGF, MMP-1 and MMP-7 were significantly higher in the lung cancer tissues compared with the adjacent normal tissues (Table I).

Association between CCR9 expression and clinical parameters in NSCLC. The CCR9 positive rate was higher in AC than SCC tissues. Additionally, CCR9 positivity was also significantly higher in the presence of lymph node metastasis. Moreover, CCR9 positive rates were significantly higher in VEGF, MMP-1 or MMP-7-positive tissue, compared with VEGF, MMP-1 or MMP-7-negative tissue, respectively. However, no significant difference in CCR9 expression was observed when patients were stratified by sex or age (Table II).

CCL25 induces the migration and invasion of NSCLC cells. A549 and SK-MES-1 cells lines demonstrated increased proliferative ability compared with the control (P<0.05) when treated with 100 ng/ml recombinant CCL25. Both A549 and SK-MES-1 cells were highly responsive in both migration and invasion assays (Fig. 2). Notably, the chemo-attractant effect of CCL25 was specific to CCR9 as demonstrated by the anti-CCR9 antibody blocking the chemo-attractant effect (Fig. 2; P<0.05).

CCL25 modulates VEGF-C, VEGF-D, MMP-1 and MMP-7 expression in NSCLC cells. At the transcriptional and protein

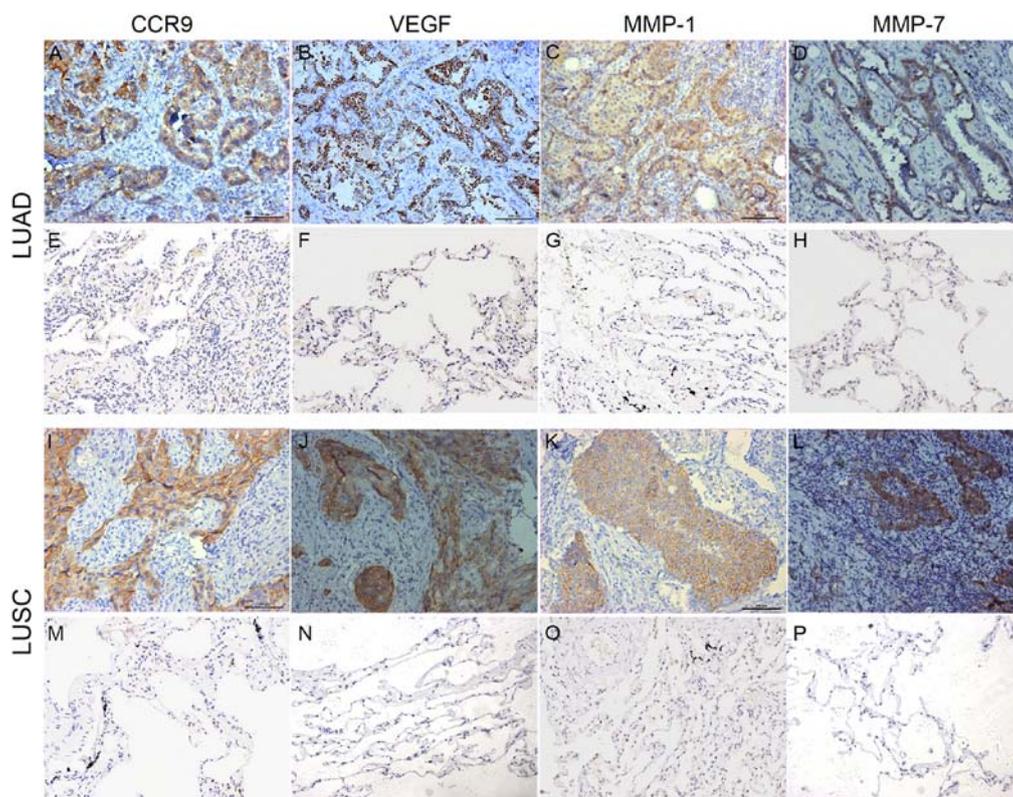


Figure 1. CCR9, VEGF, MMP-1 and MMP-7 expression in the non-small cell lung cancer and adjacent normal tissues. Positive (A) CCR9, (B) VEGF, (C) MMP-1 and (D) MMP-7 expression in LUAD tissue. Negative (E) CCR9, (F) VEGF, (G) MMP-1 and (H) MMP-7 expression in lung adjacent normal tissue. Positive (I) CCR9, (J) VEGF, (K) MMP-1 and (L) MMP-7 expression in LUSC tissue. Negative (M) CCR9, (N) VEGF, (O) MMP-1 and (P) MMP-7 expression in lung adjacent normal tissue. Magnification x200. CCR9, CC chemokine receptor 9; VEGF, vascular endothelial growth factor; MMP-1, matrix metalloproteinase-1; MMP-7, matrix metalloproteinase-7; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

level, the expressions of VEGF-C, VEGF-D, MMP-1 and MMP-7 were upregulated in the A549 and SK-MES-1 cell lines after CCL25 stimulation (Figs. 3 and 4; $P < 0.05$ in all cases). Furthermore, CCL25-induced upregulation was reversed by anti-CCR9 antibody treatment (Figs. 3 and 4; $P < 0.05$ in all cases). However, MMP-7 protein expression was not modified by CCL25 or anti-CCR9 antibody in the SK-MES-1 cells ($P > 0.05$).

Survival analysis of CCR9 and CCL25 in patients with NSCLC.

The association between CCR9 and CCL25 positive rates, and clinical prognosis in patients with NSCLC ($n=1,926$) was examined using a Kaplan-Meier analysis. Patients with lower CCR9 expression demonstrated better overall survival (OS), compared with those with higher CCR9 expression (HR=1.14; $P=0.042$; Fig. 5A). In addition, patients with lower CCL25 expression had better OS, compared with those with higher CCL25 expression (HR=1.13; $P=0.05$; Fig. 5B). Although the results presented in Fig. 5A are only marginally statistically significant, the actual OS benefits of low CCR9 and CCL25 expression in months are substantial. Indeed, in the case of CCR9, the OS benefit for high expression, compared with low expression, was as follows: i) 50 months, 357 compared with 471; ii) 100 months, 72 compared with 131; iii) 150 months, 11 compared with 46; and iv) 200 months, 1 compared with 6. In the case of CCL25, the OS benefit for high expression, compared with low expression, was as follows: i) 50 months, 354 compared with 474; ii) 100 months, 80 compared with

123; iii) 150 months, 14 compared with 43; and iv) 200 months, 1 compared with 6. Moreover, in a sub-group analysis, low levels of CCR9 mRNA were significantly associated with an improved OS for patients who never smoked (Fig. 5C; HR=2.22; $P < 0.01$). However, there was no similar association between the expression of CCL25 mRNA and OS among patients who never smoked (Fig. 5D; HR=1.49; $P=0.16$). Furthermore, low levels of both CCR9 and CCL25 mRNA were indicative of a favorable OS outcome among patients who smoked (Fig. 5E and F).

Mutation analysis.

The mutation status of CCR9 and CCL25 in lung tumor tissues was examined using the public database of cBioPortal for Cancer Genomics (Fig. 6). As presented in Fig. 6B, CCR9 had a somatic mutation rate of 0.26% in NSCLC (3/1,144) in a TCGA 2016 study (18), 0.55% in lung AC (1/183) in the Imielinski *et al* (19) study. For lung SCC, the CCR9 mutational rates were found to be 0.62 (3/487), 0.56 (1/178) and 0.2% (1/511) in TCGA PanCan (20), TCGA pub (21) and TCGA studies (http://gdac.broadinstitute.org/runs/stddata_2016_01_28/data/LUSC/20160128/gdac.broadinstitute.org_LUSC.Mutation_Package_Calls.Level_3.2016012800.0.0.tar.gz), respectively.

CCL25 had a somatic mutation rate of 0.17% (2/1,144) in NSCLC data from TCGA 2016 study and 0.53% (3/566) in lung AC data from TCGA PanCan study (Fig. 6C). Altogether, these results suggested that mutation of CCR9 and CCL25 is

Table I. Association between CCR9 and clinicopathological characteristics.

Clinicopathological characteristic	Number of patients	CCR9 positivity [N, (%)]	χ^2	P-value
Sex				
Male	40	25 (62.50)	0.330	0.566
Female	20	14 (70.00)		
Age (years)				
≤ 60	37	22 (59.46)	1.302	0.254
> 60	23	17 (73.91)		
Histology				
SCC	25	12 (48.00)	5.444	0.020
AC	35	27 (77.14)		
Lymph node metastasis				
No	15	4 (26.67)	12.918	<0.001
Yes	45	35 (77.78)		
VEGF				
Positive	31	25 (80.65)	6.901	0.008
Negative	29	14 (48.28)		
MMP-1				
Positive	36	27 (75.00)	3.956	0.047
Negative	24	12 (50.00)		
MMP-7				
Positive	33	29 (87.88)	16.873	<0.001
Negative	27	10 (37.04)		

SCC, squamous cell carcinoma; AC, adenocarcinoma; CCR9, CC chemokine receptor 9; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase.

Table II. Expression of CCR9, VEGF, MMP-1 and MMP-7 in tumor tissues and adjacent normal tissues in patients with non-small cell lung cancer.

Parameter	Tumour tissue, n (%)	Adjacent normal tissue, n (%)	χ^2	P-value
CCR9	39 (65.00)	15 (25.00)	19.394	<0.0001
VEGF	31 (51.67)	10 (16.67)	16.338	<0.0001
MMP-1	36 (60.00)	11 (18.33)	21.860	<0.0001
MMP-7	33 (55.00)	6 (10.00)	27.693	<0.0001

CCR9, CC chemokine receptor 9; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase.

a rare occurrence in patients with NSCLC. This is in stark contrast with epidermal growth factor receptor (EGFR), which has a mutational rate of ~10% in Caucasian patients with NSCLC and $\leq 50\%$ of Asian patients with NSCLC (22-24), or ALK receptor tyrosine kinase (ALK) with a 3-5% rate in patients with NSCLC (25).

Discussion

In the present study, the CCL25/CCR9 signaling axis was demonstrated to regulate the expression of VEGF-C, VEGF-D, MMP-1 and MMP-7, and may promote the invasion and migration of the lung cancer cells. Survival analysis demonstrated

that patients with lower expression of CCL25 or CCR9 in their tumors displayed better prognosis.

Chemokines are known mediators of leukocyte trafficking and host defense (26). Indeed, previous studies have determined that the involvement of chemokine receptors is of importance in patient prognosis (27), apoptosis (28) and metastatic (29) signaling machinery in various cancer types. Among all the chemokine receptors, studies on the role of CXCR4 have been more extensive. These previous studies suggested that CXCR4 (8,12,30-32) was highly expressed in NSCLC, and functional blockade of this interaction could inhibit metastasis to the bone marrow, lymph nodes or pleural space. These findings highlight the effect of

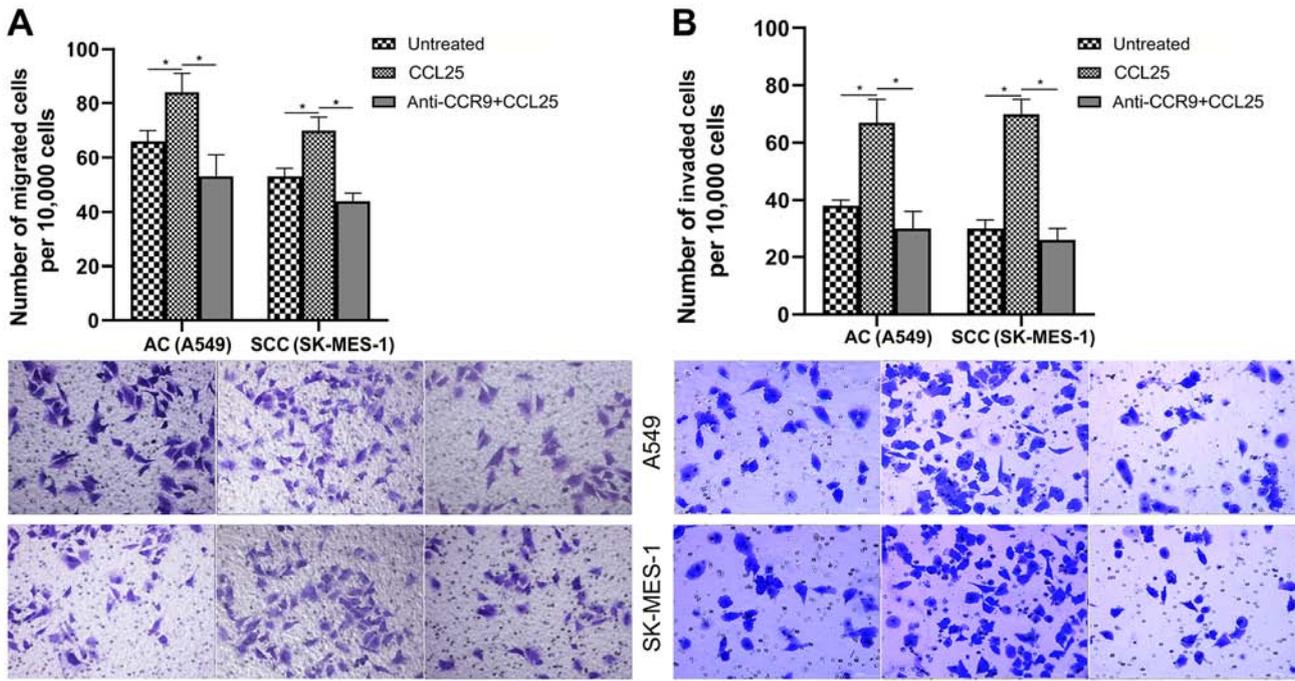


Figure 2. Migration and invasion of A549 and SK-MES-1 cell lines. (A) Number of migrating cells was significantly increased after CCL25 treatment and the addition of an anti-CCR9 antibody could significantly abrogate this increase. (B) Number of invading cells was significantly increased after CCL25 treatment and the addition of an anti-CCR9 antibody significantly inhibited this increase. Magnification, x200. *P<0.05. CCR9, CC chemokine receptor 9; CCL25, CC motif chemokine ligand 25; AC, adenocarcinoma; SCC, squamous cell carcinoma.

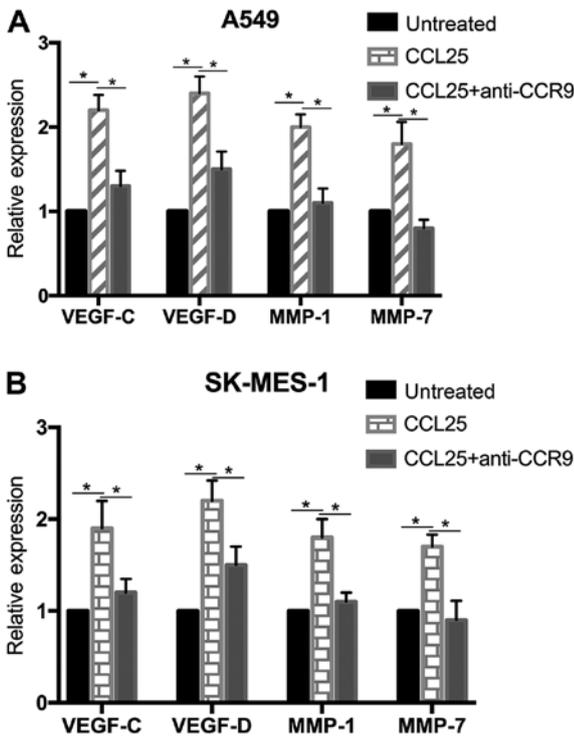


Figure 3. mRNA expression of VEGF-C, VEGF-D, MMP-1 and MMP-7 in non-small cell lung cancer cell lines. (A) Expression of VEGF-C, VEGF-D, MMP-1 and MMP-7 in A549 cells significantly increased after CCL25 treatment, and the addition of an anti-CCR9 antibody inhibited CCL25-mediated upregulation. (B) Expression of VEGF-C, VEGF-D, MMP-1 and MMP-7 in SK-MES-1 cells increased after CCL25 treatment, and the anti-CCR9 antibody abrogated this CCL25-mediated increase. *P<0.05. CCR9, CC chemokine receptor 9; CCL25, CC motif chemokine ligand 25; VEGF, vascular endothelial growth factor; MMP-1, matrix metalloproteinase-1; MMP-7, matrix metalloproteinase-7.

chemokine/chemokine receptor signaling on the metastatic potential of NSCLC.

Several steps are required to achieve metastasis, including active migration, extracellular matrix degradation and adhesion to vascular endothelial cells. Migration and invasion associated with metastatic potential can be triggered by chemokine binding to chemokine receptor on the cell surface (33,34). Tumor lymphangiogenesis was previously found to be associated with the VEGF-C/VEGF-D/VEGF receptor-3 (VEGFR-3) signaling axis (35,36). Previous studies have determined VEGF-C activates VEGFR-3, which in turn promotes proliferation (37), migration (38) and apoptosis protection (39). In various cancer types, the tumor cells produce VEGF-C and recruit monocytes or macrophages into tumor tissue (40). These monocytes and macrophages differentiate to M2-polarized tumor-associated macrophages, which also produce VEGF-C, and further increase lymphatic vessel development (41). Moreover, lymph-angiogenic factors derived from normal lymphatic cells can reprogram the gene expression profile of these cells and convert them to tumor-derived lymphatic cells during tumor development and progression (42). Tumor-derived lymphatic cells express specific lymphatic markers, such as VEGFR-3 and lymphatic vessel endothelial receptor 1, and form a lymphatic system *in vivo* (43).

VEGF-C and VEGF-D, members of the VEGF family, have been demonstrated to stimulate the proliferation of lymphatic endothelial cells, and to promote lymphatic invasion and lymph node metastasis through VEGFR-3 signaling, which is critical for the growth of lymphatic vessels (44,45). These findings provided theoretical evidence for the role of VEGF-C and VEGF-D in promoting cancer metastasis (46).

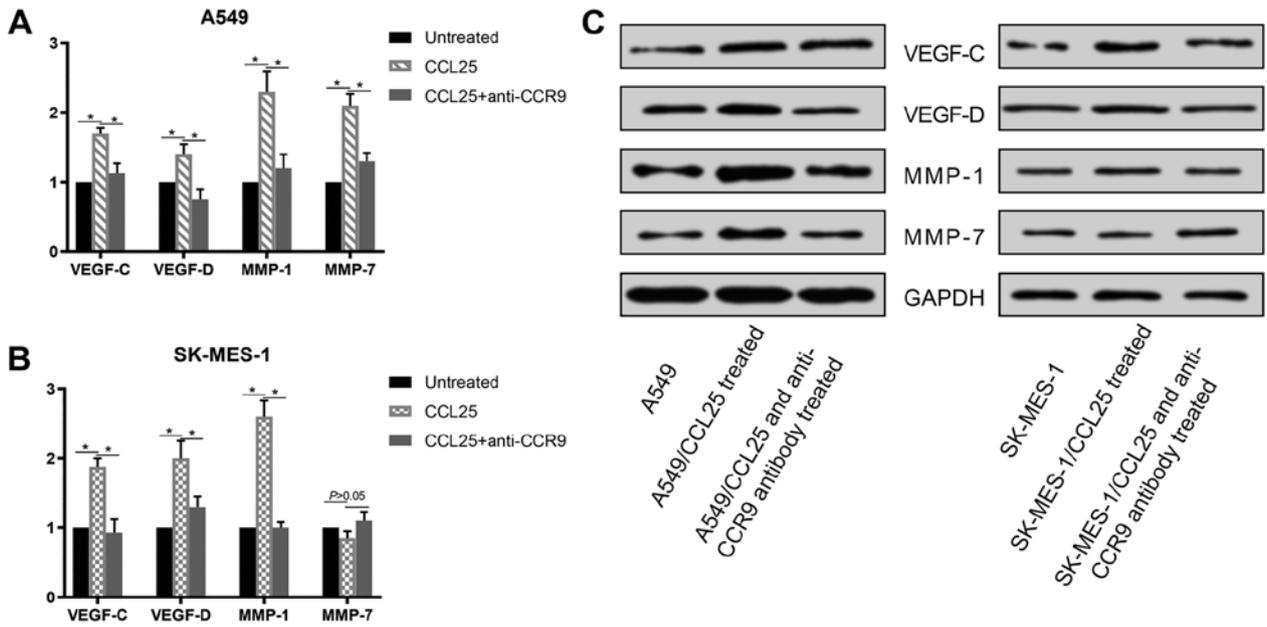


Figure 4. Protein expression of VEGF-C, VEGF-D, MMP-1 and MMP-7 in non-small cell lung cancer cell lines. (A) Expression of VEGF-C, VEGF-D, MMP-1 and MMP-7 in A549 cells increased following CCL25 treatment and the addition of an anti-CCR9 antibody abrogated this upregulation. (B) Expression of VEGF-C, VEGF-D and MMP-1 in SK-MES-1 cells increased following CCL25 treatment. Addition of anti-CCR9 could abrogate this increase. (C) Protein expression of VEGF-C, VEGF-D, MMP-1 and MMP-7 was examined in A549 and SK-MES-1 cells. *P<0.05. CCR9, CC chemokine receptor 9; CCL25, CC motif chemokine ligand 25; VEGF, vascular endothelial growth factor; MMP-1, matrix metalloproteinase-1; MMP-7, matrix metalloproteinase-7.

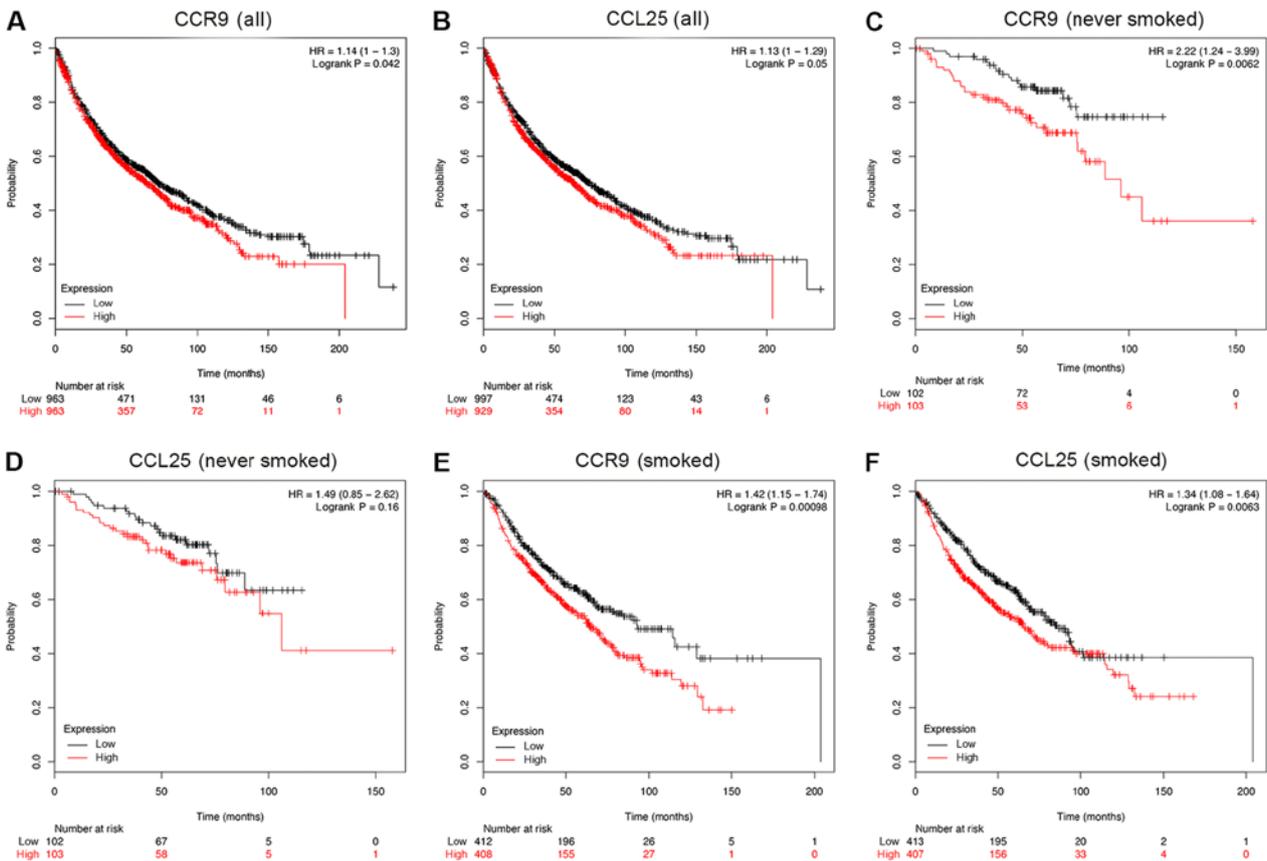


Figure 5. Survival analysis of patients with NSCLC according to CCL25 and CCR9 expression. Kaplan-Meier survival plots showing that high expression of CCR9 or CCL25 is associated with poor survival in patients with NSCLC. (A) Association between CCR9 mRNA expression and OS. (B) Association between CCL25 mRNA expression and OS. (C) Association between CCR9 mRNA expression and OS among patients who never smoked. (D) Association between CCL25 mRNA expression and OS among patients who never smoked. (E) Association between CCR9 mRNA expression and OS among patients who smoked. (F) Association between CCL25 mRNA expression and OS among patients who smoked. NSCLC, non-small cell lung cancer; CCR9, CC chemokine receptor 9; CCL25, CC motif chemokine ligand 25; OS, overall survival.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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