

CX3CL1 promotes lung cancer cell migration and invasion via the Src/focal adhesion kinase signaling pathway

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Abstract. The present study investigated the role of C-X3-C motif chemokine ligand 1 (CX3CL1) in lung cancer cell migration and invasion and its potential mechanism. The expression levels of C-X3-C motif chemokine receptor 1 (CX3CR1) in six human lung cancer cell lines and one human bronchial epithelial cell line were assessed using reverse transcription-quantitative polymerase chain reaction and western blotting. Cell proliferation was assessed using the Cell Counting Kit-8 assay. Cell migration and invasion were examined using the Transwell assay, with and without Matrigel, respectively. The signaling pathway activated by CX3CL1 was analyzed via western blotting and inhibitory migration and invasion assays. CX3CR1 was expressed in the six lung cancer cell lines and one normal lung cell line. The lung cancer cell line, H460, was selected for further study. CX3CL1 did not significantly affect H460 proliferation; however, CX3CL1 did significantly enhance the migration and invasion of H460 cells. The Src/focal adhesion kinase (FAK) signaling pathway was activated in a time-dependent manner upon stimulation of CX3CL1. However, blocking Src activity with saracatinib prevented CX3CL1-mediated cell migration and invasion. Therefore, the findings indicated that CX3CL1 promotes lung cancer cell migration and invasion *in vitro*, and the Src/FAK signaling pathway serves a vital role in this process.

Introduction

The lungs are the leading site of cancer in males. Notably, lung cancer contributes to 17% of new cancer cases and 23% of cancer fatalities (1). Due to increases in cigarette smoking and environmental pollution, novel cases and lung cancer-associated mortalities have increased in China (2). Surgery and chemotherapy are two major treatment strategies

for patients with lung cancer, and are performed according to their pathological type (3). However, advancements in therapy have led to improvements in overall survival, and more lung cancer-associated fatalities are ascribed to distant metastases rather than the primary tumor (4). The treatment of metastatic lung cancer has represented a challenge for clinicians and researchers. Unfortunately, the molecular mechanisms underlying lung cancer metastases remain poorly understood. This knowledge gap prevents the development of a potential marker and therapeutic target for lung cancer prediction and treatment, respectively.

Chemokines are classified into four highly conserved groups according to the mutual arrangements of cysteine residues and disulfide bridges: CXC, CC, C and CX3C (5). As the name suggests, the CX3C subgroup contains conserved cysteine residues at positions 8, 12, 34 and 50 in humans and other species (6). C-X3-C motif chemokine ligand 1 (CX3CL1), the only member of the CX3C subgroup, is a transmembrane protein, containing a mucin-like stalk with a chemokine domain on the top and a short intracellular C terminus on the bottom (7,8).

Since the stalk in this setting appears to serve as a domain extender, the chemokine domain assumes the CX3CL1-C-X3-C motif chemokine receptor 1 (CX3CR1) interaction. There are two forms of CX3CL1, the membrane-attached form and the shed form. Multiple cleavage sites exist in the CX3CL1 stalk structure, which results in multiple distinct shed forms of this chemokine (9). Furthermore, the various shed forms of CX3CL1 may be associated with the specific cell types within the different tissues (10).

As indicated by various studies, the CX3CL1-CX3CR1 interaction is involved in various clinical diseases, including cancer (11,12). However, reports on the clinical role of CX3CL1 in tumors are contradictory because CX3CL1 exerts pro-tumor and antitumor effects. The CX3CL1-CX3CR1 interaction has demonstrated pro-tumor effects in multiple types of cancer, including breast cancer (12,13), B-cell lymphoma, colon (14), ovarian (15), prostate (16), pancreatic (17) and renal cell cancer (18). This discrepancy may be ascribed to the dual functions of CX3CL1 as a chemoattractant for leukocytes and an adhesion molecule for tumor cells. However, there are relatively few studies on the effects of CX3CL1 on lung cancer, let alone the molecular mechanism involved.

In the present study, it was investigated whether CX3CR1 was expressed by the lung cancer cell lines. Experiments were

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performed to investigate the role of CX3CL1 in the proliferation and movement of lung cancer cells. Furthermore, the molecular mechanism was also studied. The findings provide a basis for further study in this field.

Materials and methods

Cell lines and cell culture. Six human lung cancer cell lines (H1650, H292, H460, A549, HCC827, SK-MES-1) and one human bronchial epithelial cell line (BEAS-2B) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere (5% CO₂/95% air).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total cellular RNA from the cells. Following this, the total RNA was converted to cDNA with the RT reagent Kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Subsequently, RT-qPCR was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). All primers were purchased from Sangon Biotech Co. Ltd. (Shanghai, China) and the sequences were as follows: β -actin, sense 5'-CAACCGCGAGAAGATGAC CC-3' and antisense 5'-GAGGCGTACAGGGATAGCAC-3'; CX3CR1, sense 5'-AGTGTCACCGACATTTACCTCC-3' and antisense 5'-AAGGCGGTAGTGAATTTGCAC-3. qPCR was performed using SYBR-Green Mix (Takara Bio, Inc.). The following PCR conditions were used: Initial denaturation, 1 cycle of 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 60°C for 34 sec. Relative quantification of the aforementioned genes was determined using the 2^{- $\Delta\Delta C_q$} method with β -actin as an endogenous control (19).

Immunofluorescence microscopy. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Following this, cells were rinsed three times with PBS, overlaid with 5% protease-free bovine serum albumin (Sangon Biotech Co. Ltd.) for 1 h at room temperature, rinsed with PBS and incubated with primary antibody against CX3CR1 (1:1,000, cat. no. ab8021, Abcam, Cambridge, MA, USA) at 4°C overnight. Cells were incubated with fluorescein-conjugated goat anti-rabbit IgG antibody (1:1,000, cat. no. ab150077, Abcam, Cambridge, MA, USA) for 1 h at room temperature following washing with PBS. Subsequently, cells were washed three times with PBS and mounted in Hoechst solution (cat. no. ab228551; Abcam, Cambridge, MA, USA) for 0.5 h at room temperature. Slides were viewed with a confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Western blotting. Cells were harvested at 80% confluence using lysis buffer with phosphatase and protease inhibitor cocktails (Cell Lysis Buffer; Cell Signaling Technology, Inc., Danvers, MA, USA). The total protein was detected using BCA methods. Cell lysates containing equivalent amounts of proteins

(30 μ g/lane) were subjected to SDS-PAGE using 10% polyacrylamide gels. Following this, the proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline with Tween-20 for 1 h at room temperature. Monoclonal antibodies recognizing CX3CR1 (cat. no. WH0001524M1; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and Tubulin (cat. no. AT819; Beyotime Institute of Biotechnology, Haimen, China) were used to blot the membrane at 4°C overnight (all 1:1,000). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (cat. no. A0208/A0216; Beyotime Institute of Biotechnology) were used as the secondary antibodies to detect the primary antibody for 2 h at room temperature (1:500). The bands were detected using chemiluminescence reagents (Thermo Fisher Scientific, Inc.). ImageJ 1.51m (National Institutes of Health, Bethesda, MD, USA) was used to analyze the bands.

Notably, preliminary experiments with 20-200 nM CX3CL1 treatment (PeproTech, Inc., Rocky Hill, NJ, USA) demonstrated that 100 nM CX3CL1 produced the strongest effect. Therefore, for CX3CL1-induced signaling, cells were stimulated with 100 nmol/l of CX3CL1 for 15, 30 and 60 min. For signaling pathway inhibition, cells were pre-treated with 100 nmol/l of the Src inhibitor, saracatinib (Selleck Chemicals, Houston, TX, USA), for 1 h prior to stimulation with the same amount of CX3CL1 for 15, 30 and 60 min. Monoclonal antibodies for the unphosphorylated and phosphorylated forms of Src (phosphorylation of tyrosine 416) and focal adhesion kinase (FAK) (phosphorylation of tyrosine 576/577) were purchased from Cell Signaling Technology, Inc., to evaluate the activation status of the Src/FAK signaling pathway. Anti-GAPDH (Beyotime Institute of Biotechnology) was used to detect GAPDH, which served as the internal reference.

Cell counting Kit-8 (CCK-8). Cells were seeded onto a 96-well plate at 2x10³ cells/well. Following 24 h of incubation, the cells were stimulated with 50 nmol/l of CX3CL1 in the experimental group. In the control group, the same amount of distilled water was added to the wells. After 1, 2 and 3 days, CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to the corresponding wells, and the plate was incubated at 37°C for 2 h. A multi-well spectrophotometer was used to measure the absorbance at 450 nm.

In vitro migration and invasion assay. The migration and invasion assays were performed using 24-well Transwell chambers with 8- μ m pores (Corning Incorporated, Corning, NY, USA); however, the chambers were coated with Matrigel for the invasion assay.

Briefly, after extensive washing, 3x10⁴ (migration assay) or 6x10⁴ (invasion assay) cells were suspended in DMEM without FBS and plated on each upper chamber. The lower chambers were filled with DMEM containing 0.1% bovine serum albumin, which was supplemented with or without 100 nmol/l of CX3CL1. For inhibition of the assays, the upper chambers were treated with 100 nmol/l of saracatinib for 1 h prior to the assays. Following 24 h of incubation at 37°C, the cells in the upper chamber of the membrane were removed with a cotton swab, and the cells on the underside were fixed with paraformaldehyde and stained with 0.1% crystal violet.

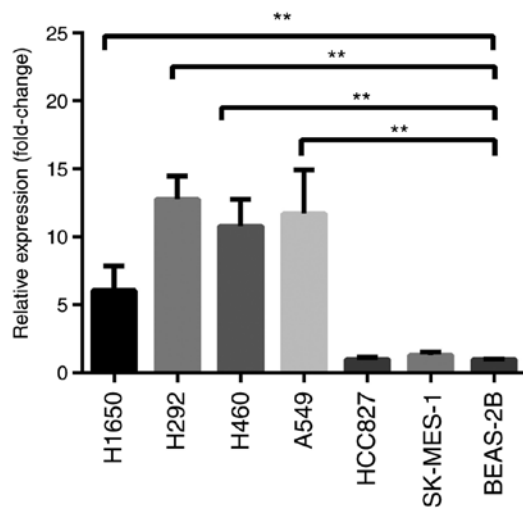


Figure 1. Expression of CX3CR1 mRNA in cell lines. H1650, H292, H460, A549 contained higher levels of CX3CR1 mRNA than BEAS-2B. Data were presented as the mean \pm standard deviation (N=3). **P<0.01 as indicated. CX3CR1, C-X3-C motif chemokine receptor 1.

The cells were counted in three randomly selected fields under light microscope (magnification, x40).

Statistical analysis. Data were expressed as the mean \pm standard deviation. Significant differences were identified using the one-way analysis of variance with subsequent use of post hoc LSD tests to differentiate between two groups when needed.

P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of CX3CR1 in the cell lines. RT-qPCR and western blotting were used to analyze CX3CR1 mRNA and protein expression levels, respectively. RT-qPCR results revealed that the CX3CR1 mRNA levels were significantly increased in the H1650, H292, H460 and A549 cells compared with BEAS-2B cells (Fig. 1). The protein expression levels of CX3CR1 were significantly increased in all six lung cancer cell lines when compared with BEAS-2B (Fig. 2). Notably, the two CX3CR1 bands exhibited in Fig. 2A can be attributed to various factors. Firstly, CX3CR1 may have isomers. Secondly, there was slight degradation of CX3CR1 during detection. The upper band with the anticipated molecular mass was used to calculate the expression. Notably, there was a discrepancy between the expression levels of CX3CR1 mRNA and protein. Based on the data from both evaluations, H460 was selected for further study. Furthermore, the cell membrane was not permeated by 0.1% Triton X-100. Therefore, the signal represented the cell surface CX3CR1 expression (Fig. 2C).

Impact of CX3CL1 on tumor cell proliferation. To investigate whether CX3CL1 has a direct impact on tumor cell proliferation, the CCK-8 assay was used to evaluate the growth of H460 with and without CX3CL1. As indicated in Fig. 3, CX3CL1 did not significantly affect the proliferative capacity of H460.

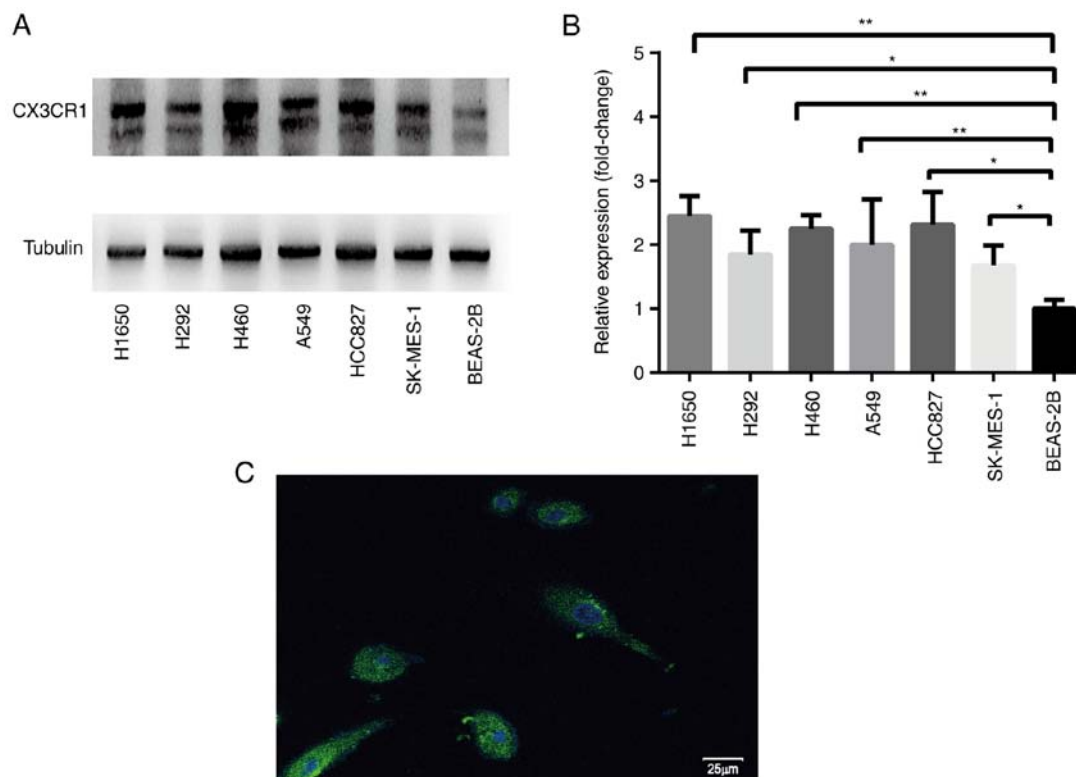


Figure 2. Expression of CX3CR1 protein in cell lines. According to the molecular mass of CX3CR1, the upper band in CX3CR1 was used to calculate expression. (A and B) CX3CR1 was highly expressed in the lung cancer cell lines compared with BEAS-2B. (C) Cell surface CX3CR1 expression was detected using the immunofluorescence combined with Hoechst staining for H460 nuclei. Data were presented as the mean \pm standard deviation (N=3). *P<0.05 and **P<0.01 as indicated. CX3CR1, C-X3-C motif chemokine receptor 1.

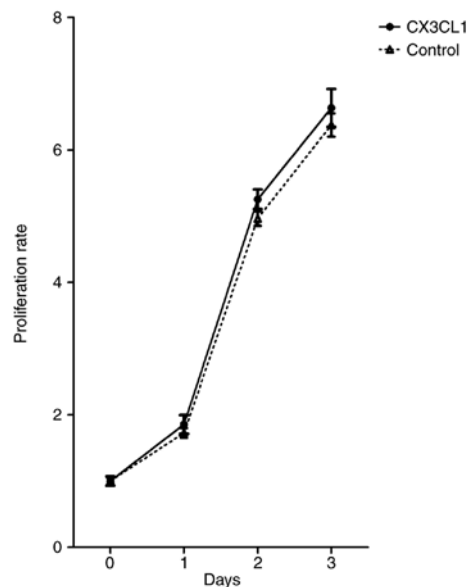


Figure 3. Proliferation rate of H460 with and without CX3CL1. There was no significant difference in the proliferation rate between the two groups. Data were presented as the mean \pm standard deviation (N=3). CX3CL1, C-X3-C motif chemokine ligand 1.

Impact on tumor cell movement. As indicated in Fig. 4, Transwell invasion and migration assays were performed. Compared with the cells without stimulation of CX3CL1, the migration

and invasion abilities of H460 were significantly increased following 24 h of exposure to CX3CL1 (Fig. 4A-C and G). However, saracatinib significantly inhibited the enhanced CX3CL1-induced migration and invasion abilities of H460 to lower than basal level (Fig. 4D-F and H). Notably, CX3CL1 did not elicit a significant response in H292 and A549 cells (data not shown).

CX3CL1-induced activation of the Src/FAK pathway. To obtain evidence that CX3CL1 promoted H460 cell migration and invasion via the Src/FAK signaling pathway, the phosphorylation of Src and FAK was analyzed in CX3CL1-stimulated cells. In this experiment, CX3CL1-induced Src phosphorylation peaked 15 min post-stimulation. In addition, CX3CL1-induced FAK phosphorylation peaked 30 min post-stimulation (Fig. 5A). However, saracatinib hindered CX3CL1-induced Src and FAK phosphorylation (Fig. 5B).

Discussion

Although the CX3CL1-CX3CR1 interaction is well known in the metastatic process of variety of cancer types, the present study provided insight into the role of CX3CL1 as an enhancer of metastasis in lung cancer. The present findings provided evidence that CX3CL1 promotes the chemotaxis ability of lung cancer cells by binding to CX3CR1 and activating the Src/FAK signaling pathway.

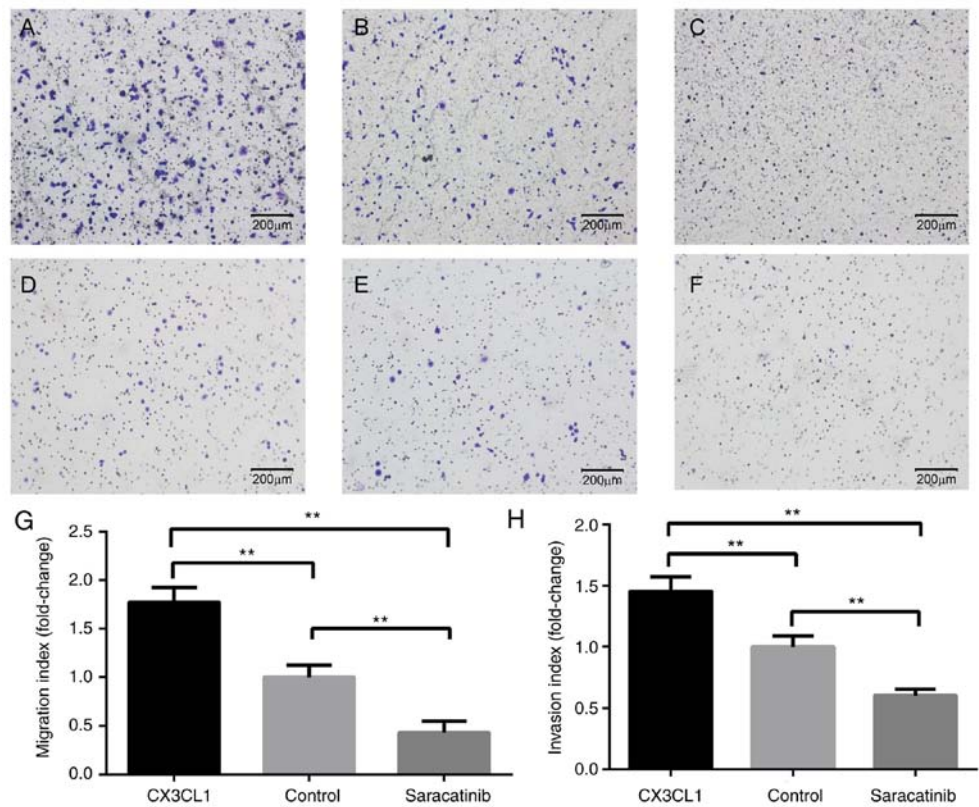


Figure 4. Cell invasion and migration assays. Representative images of H460 cell migration, following treatment (A) with or (B) without CX3CL1, were captured (magnification, x100). (C) H460 was incubated with saracatinib during CX3CL1-stimulated migration. Representative images of H460 cell invasion, following treatment (D) with and (E) without CX3CL1, were captured (magnification, x100). (F) H460 was incubated with saracatinib during CX3CL1-stimulated invasion. Quantitative results for the (G) migration and (H) invasion assays were indicated. The migration and invasion indices were calculated as the number of migrated cells compared to that of control group. Data were presented as the mean \pm standard deviation (N=3) **P<0.01 as indicated. CX3CL1, C-X3-C motif chemokine ligand 1.

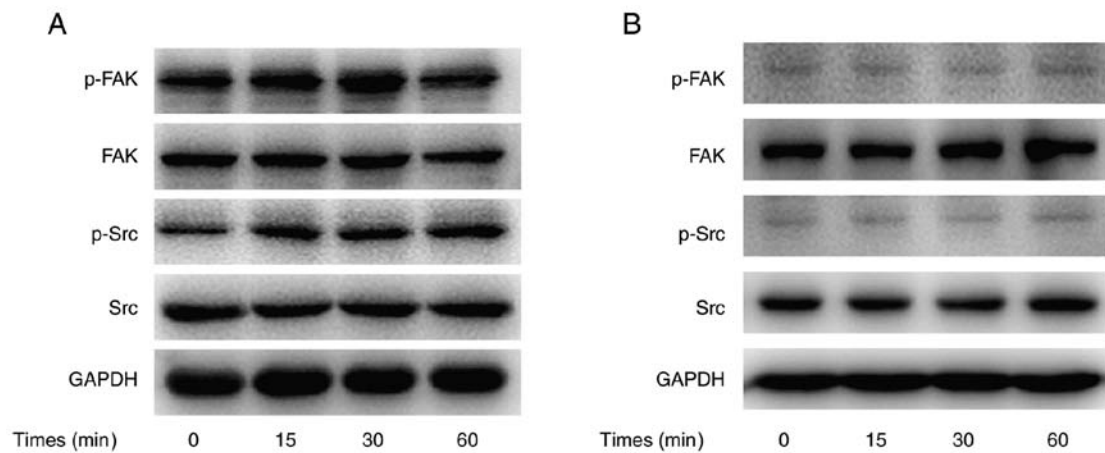


Figure 5. CX3CL1 activates the Src/FAK signaling pathway in H460. CX3CL1 induced (A) Src and FAK phosphorylation, which peaked at either 15 min or 30 min after stimulation, respectively. (B) Pre-treatment of H460 with Saracatinib abrogated Src and FAK phosphorylation. FAK, focal adhesion kinase; CX3CL1, C-X3-C motif chemokine ligand 1.

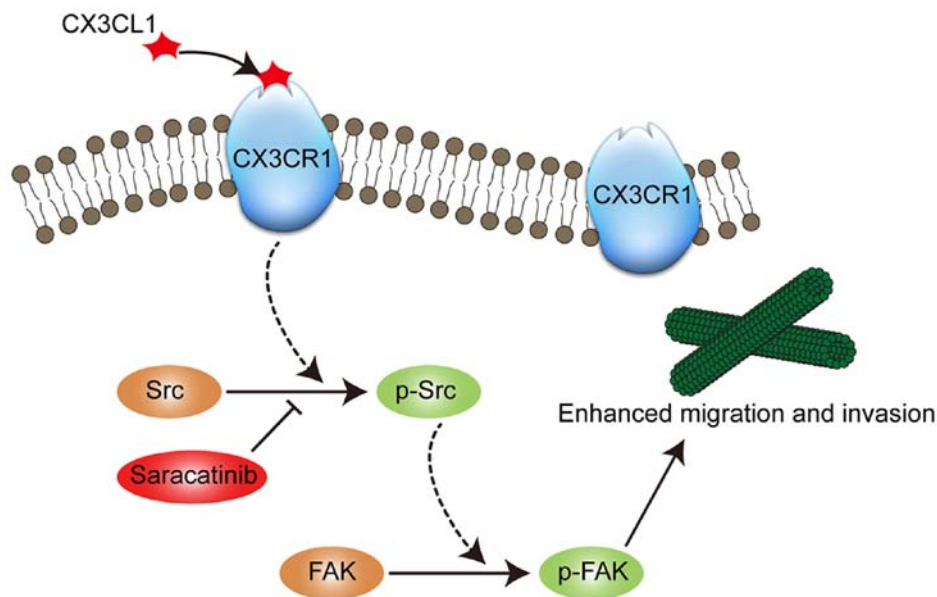


Figure 6. Model of CX3CL1-enhanced lung cancer cell chemotaxis ability. CX3CL1 may promote the chemotaxis ability of lung cancer cells by enhancing the Src/FAK signaling pathway. Saracatinib could abrogate the effect of CX3CL1 on chemotaxis. CX3CL1, C-X3-C motif chemokine ligand 1; CX3CR1, C-X3-C motif chemokine receptor 1; FAK, focal adhesion kinase.

The precursor of CX3CL1 is synthesized as an intracellular 50-75 kDa protein that is rapidly processed and transported to the cell surface (20). A soluble 85-kDa fragment of CX3CL1, containing a 76-amino acid chemokine domain, can be cleaved from the cell surface under normal growth conditions. However, this process may be accelerated by stimuli, such as cancer (21). Therefore, under physiological and pathological conditions, CX3CL1 is allowed to mediate the chemotaxis and firm capture of CX3CR1-expressing cells via the soluble and membrane-attached form of CX3CL1, respectively (22). CX3CR1, a seven-transmembrane G-protein-coupled receptor, mediates the activation of the downstream signaling pathway (c-Raf, mitogen-activated protein kinase kinase, extracellular signal-regulated kinase and nuclear factor- κ B) through its ligand, CX3CL1 (23).

The present study investigated CX3CR1 expression at mRNA and protein levels. Although all six lung cancer cell lines demonstrated overexpression of CX3CR1 at protein levels, some of the lung cancer cell lines were not upregulated at mRNA levels. The discrepancy between the mRNA and protein expression levels may be due to posttranscriptional and posttranslational control mechanisms. Thus, the amount and frequency of protein synthesis may not simply coincide with the amount of mRNA (24). Since H460 exhibited higher CX3CR1 expression at mRNA and protein levels, this cell line was selected for further experiments in the present study.

To study the potential effects of CX3CL1 on lung cancer cell proliferation, the proliferation rate of H460 with and without CX3CL1 was assessed using CCK-8 *in vitro*. No significant differences were observed between the two groups. However, Tardaguila *et al* (25) reported that CX3CL1

contributes to tumorigenesis in breast cancer. Through proteolytic shedding of an ErbB ligand, CX3CL1 triggered cell proliferation by transactivating the ErbB receptors. Thus, the increased tumor multiplicity was a consequence of CX3CL1 acting as a positive modifier of breast cancer in concert with ErbB receptors rather than CX3CL1-induced metastatic dissemination of the primary tumor (25). This inconformity may be associated with the different molecular mechanisms of tumor heterogeneity.

Based upon the observations from the *in vitro* migration and invasion assays in the present study, CX3CL1 significantly promoted the chemotaxis ability of lung cancer cells. Notably, the results were negative for H292 and A549 cells, which can be attributed to tumor heterogeneity. Furthermore, the present study revealed that the molecular mechanisms, following CX3CL1 activation in lung cancer cells, involved the Src/FAK signaling pathway. Src, a proto-oncogene, is a non-receptor protein-tyrosine kinase that is a critical regulator of signal transduction induced by a variety of cell-surface receptors. Src serves a key role in cell growth, division, migration and survival signaling pathways. Furthermore, Src activity is regulated by tyrosine phosphorylation of two chief sites, pTyr416 and pTyr530. Phosphorylation of Tyr416, located in the activation loop of the kinase domain, by an adjacent Src molecule results in upregulated enzyme activity. However, phosphorylation of Tyr530, located in the regulatory tail, by C-terminal Src kinase or Csk homologous kinase renders the enzyme less active (26,27). FAK is another subgroup of the non-receptor protein tyrosine kinases, and it is regulated by phosphorylation and dephosphorylation. FAK has been identified to participate, through various pathways, in a diverse spectrum of receptor-induced biological activities, particularly the activation of cell spreading and migration through integrin-mediated signal transduction (28). Specifically, integrin clustering results in the phosphorylation of Tyr397, which prepares the binding site for Src family kinases. Following this, the recruitment of Src family kinases leads to the phosphorylation of Tyr576/577 in the catalytic domain (29). Therefore, when deregulated, the Src/FAK complex indicates strong oncogenic activity. Based on the present study, a model was proposed in which CX3CL1-CX3CR1 interaction first phosphorylates Src at Tyr416. Subsequently, the phosphorylated Src binds with FAK and phosphorylates FAK at Tyr576/577 (Fig. 6). Saracatinib, a Src inhibitor, could abrogate the effect of CX3CL1 on chemotaxis. Saracatinib has also been reported to inhibit cell movement in prostate cancer (30) and bladder cancer (31), which is consistent with the present study. Therefore, saracatinib may translate as a clinical therapy that inhibits cancer metastasis; however, further studies are required.

In conclusion, CX3CL1 enhanced the migration and invasion of lung cancer cells. It was proposed that the Src/FAK signaling pathway is involved in CX3CL1-CX3CR1 interaction, which causes enhanced migration and invasion. The present results also highlight the potent anti-migratory and anti-invasive effects of saracatinib *in vitro*.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JD conceived the study and revised the manuscript. WL and YL performed the experiments and wrote the manuscript. QC and LJ collected and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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