Differential Expression of CXCR4 Is Associated with the Metastatic Potential of Human Non–Small Cell Lung Cancer Cells

Liping Su, Jinping Zhang, Huanbin Xu, Ying Wang, Yiwei Chu, Ruizi Liu, and Sidong Xiong

Abstract

Purpose: To evaluate the relation between CXCR4 expression and the presence of metastatic disease in human non–small cell lung cancer (NSCLC) patients and investigate whether modulation of CXCR4 expression could serve as a potential pathway in preventing metastasis of NSCLC.

Experimental Design: CXCR4 expression in 36 patients with NSCLC and 10 normal lung tissues was detected by real-time PCR and immunohistochemistry. CXCR4 expression in two human NSCLC clones (95C and 95D) with different metastatic potential was determined by real-time PCR and flow cytometry. 95C and 95D cells were transfected with the plasmid DNA containing CXCR4 coding gene or CXCR4 antisense nucleotide fragment, respectively, and the effects on in vitro cell migration, invasion, and adhesion and in vivo metastasis were measured.

Results: Up-regulated expression of CXCR4 was detected in 34 tumors, which were further divided into 17 high expression cancers and 17 low expression cancers by their staining intensities. High CXCR4 tumors (13 of 17) were more prone to clinical metastasis in comparison with low expression tumors. CXCR4 was differentially expressed in 95C and 95D cells with low or high metastatic potential, and the surface expression of CXCR4 were 50% up-regulated or down-regulated following the stable transfection. The metastatic potential of NSCLC in vitro, such as migration, invasion, and adhesion, were significantly enhanced or impaired. In addition, neutralizing the interactions of stromal cell–derived factor-1/CXCR4 in vitro with CXCR4–specific antibodies inhibited the CXCR4–dependent migration, invasion, and adhesion. Furthermore, s.c. inoculation of lung cancer cells with low expression of CXCR4 in nude mice showed 0- to 2-fold decrease in lung metastatic foci than that with high expression of CXCR4.

Conclusions: Differential expression of CXCR4 is associated with the metastatic potential of human NSCLC, raising the possibility that blockade of CXCR4/stromal cell–derived factor-1 interaction may lead the way to design novel therapeutic tools for the treatment of metastatic NSCLC patients.

Non–small cell lung cancer (NSCLC) is the most commonly diagnosed malignancy and the main cause of cancer-related deaths in Asian and Western populations. NSCLC frequently shows a preference for the regional lymph node, liver, contralateral lung, brain, and bone marrow (1, 2). Most deaths from NSCLC are caused by metastasis. Inhibition of metastasis of NSCLC is thought to be one of the important therapeutic strategies. The molecules involved in metastasis may be candidates for new targets in the therapy of NSCLC (3).

Tumor cell migration and metastasis share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors (4). Chemokines are a large family of small, structurally related heparin-binding proteins classified as C, CXC, CC, and CX3C subfamilies, which are central to the normal and pathologic trafficking of leukocytes. The biological effects of chemokines are thought to be mediated through interactions with G-protein-coupled receptors, whose nomenclature is simply based on the chemokine group (CC, CXC, C, or CX3C) to which its ligand(s) belongs (5, 6). Much attention has been paid to one particular member of the chemokine receptor family, termed CXCR4, because of its key role in HIV infection (7). The sudden surge of interest and subsequent growth in CXCR4 research has led to several surprising findings. Stromal cell–derived factor-1 (SDF-1), the natural ligand for CXCR4, is a member of the CXC chemokine family that has been found to recruit CD34+ hematopoietic progenitor cells, megakaryocytes, B cells, and T cells (6, 8–10). The involvement of CXCR4 and SDF-1 in these processes makes this chemokine–receptor pair of particular interest in the tumor metastasis.
Recently, the exciting report by Muller et al. (11) showed that the leukocyte chemotactic receptor CXCR4 and its ligand SDF-1 together governed the pattern of breast cancer metastasis in a mouse model, and antibodies against CXCR4 significantly inhibited lymph node and lung metastasis. There is growing evidence that CXCR4 and SDF-1 regulate migration and metastasis of a variety of cancers, including NSCLC (12, 13). Consistent with the previous studies, we next analyzed the expression of CXCR4 in NSCLC tissues with or without regional invasion, lymph node metastasis, or distant metastasis. We found that CXCR4 expression is low in normal lung tissues but high in malignant tumors with clinical metastasis. This provides us that blockade of CXCR4 might limit tumor metastasis. We then analyzed CXCR4 expression in human lung cancer cells with high or low metastasis potential. The surface CXCR4 expression was regulated by transfection of plasmid DNA containing CXCR4 coding gene or CXCR4 antisense nucleotide fragment. Such up-regulation or down-regulation of CXCR4 expression in human lung cancer cells resulted in the enhancement or impairment of cancer cell metastasis in vitro and in vivo. Our finding suggested that functional expression of CXCR4 might be a general characteristic of human NSCLC, and blockade of the interaction of CXCR4/SDF-1 may lead to design novel therapeutic tools for the treatment of metastatic NSCLC patients.

Materials and Methods

Specimens and cell lines. Tissue samples from patients with primary lung cancer were obtained, with informed consent, from No. 1 Hospital of Lianyungang City, where each specimen was confirmed by histopathologic diagnosis. 95C and 95D cell lines were subcloned from a low differentiated human large cell lung carcinoma cell line PLA-801 by Dr. Lezhen Chen (Department of Pathology, Chinese General Hospital of People’s Liberation Army, Beijing, People’s Republic of China), which were of different metastatic potential (14, 15). 95C, 95D, and the endothelial cell line ECV-304 were cultured in RPMI 1640 (Gibco-BRL, Grand Island, NY), which were of different metastatic potential (14, 15). 95C and 95D cell lines were subcloned to 3 hours. Tumor cells (4 × 10^6/mL) were added, washed with PBS thrice, and centrifuged with 1,500 rpm for 5 minutes. Supernatants were discarded and 0.1% glutaraldehyde (PBS, 100 μg/mL) was added. After fixing the cells at room temperature for 30 minutes, the plates were washed with 5% FCS/PBS. The anti-human CXCR4 mAb (50 μg/mL) was added followed by OPD substrate addition. The color was developed with diaminobenzidine. Sections were lightly counterstained with hematoxylin and mounted. Negative controls were done by replacing the primary antibody with PBS. The immunostained specimens were evaluated by two different observers without knowledge of the clinicopathologic features. If both agreed the staining intensity of cancer cells was defined as more than that of normal lung tissue, the tumor was categorized as high expression; other tumors were categorized as low expression.

Cellular ELISA. ELISA plates were pretreated with 200 μL of 0.1 mol/L NaHCO_3 containing 0.1% glutaral at room temperature for 2 to 3 hours. Tumor cells (4 × 10^6/mL) were added, washed with PBS thrice, and centrifuged with 1,500 rpm for 5 minutes. Supernatants were discarded and 0.1% glutaraldehyde (PBS, 100 μg/mL) was added. After fixing the cells at room temperature for 30 minutes, the plates were washed with 5% FCS/PBS. The anti-human CXCR4 mAb (50 μL/mL; 1:100 dilution) was added and incubated for 1 hour at 37°C. After washing, horseradish peroxidase–labeled goat anti-mouse IgG was added followed by OPD substrate addition. Absorption at 490 nm was measured in a microplate reader (Bio-Tek Instruments, Winooski, VT).

In vitro invasive assay. The invasive assay was done in 24-well cell culture chambers using inserts with 0.8-μm pore membranes precoated with Matrigel (28 μg/insert; Sigma, Saint Louis, MO). Cell suspensions (1 × 10^6/mL) were placed in the upper wells and the

![Fig. 1. Expression of CXCR4 in human normal lung and NSCLC specimens. Total RNA was extracted from human normal lung and NSCLC specimens, and CXCR4 expression in normal lung and NSCLC specimens with or without metastasis was detected by real-time PCR. A, normal lung and NSCLC specimens. B, NSCLC specimens with or without metastasis. C, cells of NSCLC specimens were resuspended and cellular ELISA was done to investigate the protein level of CXCR4 between NSCLC specimens with clinical metastasis and without metastasis. * P < 0.05.](www.aacrjournals.org)
lower wells were filled with fibroblast-conditioned medium. After incubation for 24 hours, cells on the lower surface of the membrane were stained by the H&E method and counted under a light microscope (×400).

**Zymography.** Collagenolytic activity was determined on substrate-impregnated gels as described previously (16). The transparent bands at M₀, 92,000 and 72,000 were considered positive enzymatic activities. The collagenase activity was quantified using Gel Image System (Tanon Scanning, Shanghai, People’s Republic of China).

**Flow cytometry.** Cells (1 × 10⁶) were first incubated with the specific CXCR4 mAb for 30 minutes at 4°C in 0.1% bovine serum albumin/PBS, washed twice, and then incubated with FITC-conjugated second antibody (IgG₂b, R&D Systems) for additional 30 minutes at 4°C. Then, cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA).

**Calcium mobilization assay.** Cells were labeled with 5 μmol/L fluo-3-AM (Sigma) for 30 minutes at 37°C, washed twice with D-Hank’s, and resuspended at 5 × 10⁷/mL in RPMI 1640 containing 2 mmol/L CaCl₂. Calcium flux following binding of the chemokine receptors was analyzed by adding chemokine SDF-1α at varying concentrations (0.1, 1, 10, 100, and 200 ng/mL, Peprotech, Rocky Hill, NJ). The mean fluorescence changes in intracellular calcium were measured by flow cytometry at 525 nm.

**Construction of the recombinant plasmids and transfection.** The amplified full-length CXCR4 was inserted into the pcDNA3 vector at EcoRI and XbaI sites. In addition, the CXCR4 encoding gene targeting 5'-untranslated region and partial translated region (~72 to 489 bp) was directly cloned into pUCm-T vector in clockwise direction (named pUCm-T-X4). Then, the cDNA inserts digested from pUCm-T-X4 were subcloned into pcDNA3 at EcoRV and BamHI sites in an anti-clockwise direction. The identity of recombinant plasmid pcDNA-ASX4 or pcDNA-X4 was confirmed by PCR and DNA sequencing.

95C or 95D cells were transfected with the recombinant plasmid or pcDNA3 using LipofectAMINE 2000 reagent (Life Technologies, Carlsbad, CA) as described by the manufacturer. The stable transfectants were established by G418 (800 μg/mL) screening and the CXCR4 expression on the surface was assessed by flow cytometry.

**Chemotaxis and chemoinvasion assay.** Migration and invasion assay were assayed in a 48-well chemotaxis chamber (Neuroprobe, Gaithersburg, MD) as described previously (11). Briefly, the RPMI 1640 contained 0.5% bovine serum albumin with or without 100 ng/mL SDF-1α was added into the bottom chamber. The 2 × 10⁵ cells in 50-μL volume were placed in the upper chamber, and the wells were incubated for 12 hours in a humidified incubator at 37°C. Triplicate wells were used for each data point. For neutralization studies, cells were preincubated with 50 μg/mL of the CXCR4 mAb or mouse IgG (R&D Systems) for 30 minutes at 37°C. At the end of incubation, the Transwell filters were removed and fixed followed by H&E staining. The cell number is the mean of five power fields (×100) of microscope.

**Cell adhesion assay.** Resting ECV-304 cells were exposed to 100 ng/mL SDF-1α for 15 minutes at room temperature and washed, and calcein AM (Molecular Probes, Eugene, OR)–labeled cells were added to the plate containing confluent ECV-304 cells. After incubation at 37°C for 20 minutes, nonadherent calcein AM–labeled cells were removed by careful washing with prewarmed RPMI 1640. To further determine the role of CXCR4 in lung cancer cells adhesion, tumor cells were preincubated with 50 μg/mL of the CXCR4 mAb in some cases. Cells in each well were collected and the fluorescence intensity was measured using fluorescence-activated cell sorting analysis. The results were expressed as % adherence = [(adhesive tumor cells) / (adhesive tumor cells + ECV-304 cells) × 100].

**In vivo metastasis studies.** 95C, 95C-pC, 95C-X4, 95D, 95D-pC, and 95D-ASX4 cells were harvested and washed twice in PBS before injection. Cell viability was >95% as determined by trypan blue dye exclusion. BALB/c nu/nu mice (6-8 weeks old) were s.c. injected with the above cells (8 × 10⁶ per mouse) and kept in laminar flow cabinets under specific pathogen-free conditions. After 10 weeks of cultivation,

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**Table 1. CXCR4 expression in human normal lung and NSCLC specimens**

<table>
<thead>
<tr>
<th>Lung tissue</th>
<th>CXCR4 expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Nonmalignant</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Malignant</td>
<td>2</td>
<td>34*</td>
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</table>

*P < 0.001.

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**Fig. 2.** Expression of CXCR4 protein in human normal lung and NSCLC specimens. Immunohistochemistry was done using antibodies specific for CXCR4 on normal lung and NSCLC specimens, and the staining intensities were evaluated. A, negative expression of CXCR4 in human normal lung tissue (×200); B, negative expression of CXCR4 in NSCLC specimen (×200); C and D, squamous cell carcinoma (×200); E and F, alveolar carcinoma (×400); G and H, adenocarcinoma (×200); I and J, carcinoïd (×400); D, F, H, and J, high intensities and with metastasis or regional invasion according to clinical data.
mice were sacrificed and primary tumors and lungs were collected and fixed for H&E staining.

**Statistics analysis.** Statistical analyses of the data were done with the aid of analysis programs in SPSS version 10.0 software. Differences between the treatment groups were examined by two-way ANOVA. Differences were considered statistically significant when $P < 0.05$.

### Results

**Detection of CXCR4 in non–small cell lung cancer specimens.** CXCR4 expression was first evaluated by quantitative reverse transcription-PCR in normal lung and NSCLC specimens at transcriptional level. Up-regulated expression of CXCR4 mRNA was found in NSCLC specimens (metastasis and nonmetastasis) compared with normal lung tissue (Fig. 1A). Moreover, CXCR4 mRNA was more highly expressed in NSCLC specimens with clinical metastasis than that in NSCLC specimens without metastasis (Fig. 1B), and a similar result was observed in cellular ELISA (Fig. 1C).

To further determine the expression of CXCR4 in human normal lung and NSCLC specimens, immunohistochemistry was done with specific antibody against CXCR4. Thirty-four of 36 NSCLC specimens were defined as CXCR4 protein positive, whereas 2 NSCLC specimens and 10 adjacent normal lung tissues were defined as CXCR4 protein negative (Table 1; $P < 0.05$). Typical immunostaining of CXCR4 in normal lung and NSCLC specimens was shown in Fig. 2. Positive staining of CXCR4 protein was identified in the cell membrane and/or cytoplasm of cancer cells, which excluded the possibility that infiltrating leukocyte could be CXCR4 positive and contributed to total CXCR4 levels. The expression level of CXCR4 was varied among these samples. Thirty-four NSCLC specimens were further classified into two groups according to the staining intensities in the membrane and cytoplasm: 17 cases had high expression of CXCR4 and 17 had low expression.

To elucidate the correlation between expression level of CXCR4 and clinicopathologic factors in human NSCLC, 34 malignant tumors with either high or low expression of CXCR4 were further analyzed. The relation between staining intensities

### Table 2. Clinicopathologic factors and CXCR4 staining intensities

<table>
<thead>
<tr>
<th>Feature</th>
<th>Staining intensities</th>
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<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Age</td>
<td>62.4 ± 10.0</td>
<td>59.2 ± 6.8</td>
</tr>
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<td>10</td>
<td>9</td>
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<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Pathologic type</td>
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<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Others</td>
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<td>2</td>
</tr>
<tr>
<td>Histologic grade</td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Metastasis*</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>7</td>
<td>13</td>
</tr>
<tr>
<td>No</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

* $P < 0.05$.

![Fig. 3. Expression of CXCR4 in 95C and 95D cells.](image)

The metastatic potential of 95C and 95D cells was first determined by in vitro invasive assay (A) and zymography (B). Differential expression of CXCR4 on 95C and 95D cells was further analyzed by real-time reverse transcription-PCR (C). Functional expression of CXCR4 on 95C and 95D cells was determined with calcium mobilization assay, and intracellular calcium flux in lung cancer cells stimulated with SDF-1α was detected at different time points (D). * $P < 0.05$. 

![Graphs showing](image)
and clinicopathologic factors was statistically analyzed and shown in Table 2. CXCR4 expression was independent on age, sex, pathologic type, or histologic grade ($P > 0.05$). However, in all examined cases, high expression of CXCR4 was apt to be detected in 13 lung cancer specimen with regional invasion, lymph node metastasis, or distant metastasis, which showed that high levels of CXCR4 expression correlated with the presence of metastatic disease in lung cancer patients ($P = 0.037$).

**Differential expression of CXCR4 on human non–small cell lung cancer with high or low metastatic potential.** The metastatic potential of 95C and 95D cells was first determined. 95D cells more strongly penetrated the filter than 95C cells (49.53 ± 5.83 versus 21.00 ± 3.87; Fig. 3A; $P < 0.05$). Moreover, the higher activity of matrix metalloproteinase-2, not matrix metalloproteinase-9, was detected in 95D cells compared with 95C cells by zymography (Fig. 3B; $P < 0.05$). These indicated that 95D cells had higher metastatic potential than 95C cells. Then, chemokine receptor CXCR4 expression was evaluated on 95C and 95D cells at both transcription and protein levels by real-time PCR and fluorescence-activated cell sorting. CXCR4 was detected in both cell lines and the expression of CXCR4 mRNA in 95D cells was higher than that of 95C cells (49.38% versus 21.94%; Fig. 3C). Flow cytometric analysis also confirmed stronger cell surface expression of CXCR4 on 95D (49.38%) than that of 95C cells (21.94%; Fig. 4).

To determine whether the expression of CXCR4 was functional, fluo-3-AM-loaded cells were stimulation with SDF-1α. Rapid and robust increase in intracellular calcium was elicited in a time-dependent manner, which was most striking during 25 to 30 seconds after addition of SDF-1α (Fig. 3D). SDF-1α also induced calcium influx in a dose-dependent manner, which was maximal at 100 ng/mL at concentrations of 1 to 200 ng/mL. These results indicated that CXCR4 was functional on lung cancer cells.

**Transfection of plasmid DNA containing CXCR4 coding gene or CXCR4 antisense nucleotide fragment altered CXCR4 expression in 95C and 95D cells.** To further elucidate the biological functions of CXCR4 in lung cancer, we used the overexpression and antisense technique to specifically regulate CXCR4 gene expression in 95C or 95D cells. 95C cells were transfected with plasmid DNA encoding CXCR4 full-length gene. After G418 screening for several weeks, the stable transfectant cell line 95C-X4 was established and CXCR4 expression on its surface was significantly up-regulated compared with 95C or the mock transfectant with pcDNA3 (named 95C-pC; Fig. 4, top). Conversely, 95D cells were transfected with CXCR4 antisense RNA, and the expression of CXCR4 on the stable transfectant 95D-ASX4 cells was significantly down-regulated when compared with that of 95D cells or the mock transfectant 95D-pC cells (Fig. 4, bottom).

**Regulation of CXCR4 expression by in vitro gene transfection altered the migration, invasion, and adhesion of non–small cell lung cancer.** We then investigated the effect of down-regulation or up-regulation of CXCR4 expression on the metastatic behavior of tumor cells. A prerequisite for tumor cells to invade the host tissue and extracellular matrix is their capability of active locomotion (17, 18). First, the effect of CXCR4 expression on the migration of lung cancer cells toward SDF-1α was evaluated. Up-regulation of CXCR4 expression enhanced cell migration to SDF-1α by 1.69- or 1.68-fold compared with either 95C or 95C-pC, whereas down-regulation
of CXCR4 significantly impaired the migratory response to SDF-1α by 1.93- or 1.70-fold, respectively, compared with either 95D or 95D-pC (Fig. 5A; P < 0.05).

Then, the chemoinvasion assay was further done to determine the changes in invasion ability after CXCR4 up-regulation or down-regulation. As shown in Fig. 5B, CXCR4 activation by its ligand SDF-1α resulted in an increased artificial extracellular matrix invasion of 95C-X4 cells by 1.69- or 1.61-fold compared with 95C or 95C-pC cells, whereas that of 95D-ASX4 cells was decreased 1.72- or 1.78-fold in response to SDF-1α when compared with either 95D or 95D-ASX4 cells (P < 0.05).

To extravasate to a target tissue, tumor cells must firmly arrest on vascular endothelium and then transmigrate (19). We further assessed if SDF-1α/CXCR4 interaction could induce the adhesion of CXCR4-positive cancer cells to microvascular endothelial cells. Calcein AM–labeled cells were incubated with ECV-304 in the presence or absence of exogenous SDF-1α. 95C and 95C-pC cells showed considerably low adhesion to ECV-304 cells without exogenous SDF-1α compared with 95D or 95D-pC cells. In the presence of exogenously added SDF-1α, 95C-X4 cells showed a remarkable increase in the adhesion to ECV-304 cells compared with 95C or 95C-pC cells (Fig. 5C; P < 0.05). SDF-1α also induced higher adhesion of 95D or 95D-pC cells to ECV-304 cells than 95D-ASX4 cells (Fig. 5C; P < 0.05).

Blocking CXCR4 by neutralizing antibody inhibited the migration, invasion, and adhesion of non–small cell lung cancer. To further determine the role of CXCR4/SDF-1α interaction in the metastasis of human lung cancer, 95C, 95C-X4, 95D, or 95D-ASX4 cells were preincubated with neutralizing CXCR4 antibody, and the migration, invasion, and adhesion of the above cells in response to SDF-1α were evaluated. Neutralization of binding to CXCR4 by CXCR4 mAb inhibited the up-regulation of migration, invasion, and adhesion elicited by SDF-1α, whereas control mouse IgG did not have a significant effect (Fig. 6A-C; P < 0.05). Therefore, increased ability of metastasis-associated behavior, such as migration, invasion, and adhesion, was due to the engagement of CXCR4 by SDF-1α, which implied the interaction of CXCR4/SDF-1α played an active role in the metastasis of lung cancer.

Regulation of CXCR4 expression on 95C and 95D cells controlled metastatic potential in nude mice. Having established that lung cancer cells expressing active CXCR4 had high metastatic potential in vitro, we further determined the contribution of SDF-1/CXCR4 interactions to metastasis in vivo in the spontaneous metastasis models of NSCLC in nude mice. 95C, 95C-pC, 95C-X4, 95D, 95D-pC, or 95D-ASX4 cells were separately inoculated into nude mice s.c. 10 weeks later, and numerous metastasis foci were found in the lungs of all nude mice with 95D and 95D-pC cells but only for two of five nude mice injected with 95D-ASX4 cells. None of the nude mice with 95C and 95C-pC cells showed spontaneous metastasis to lung, whereas three of five nude mice with 95C-X4 cells showed visible metastasis foci in lung (Fig. 7B). The metastasis foci were then counted under microscope, and there was a 2.0-fold decrease in the number of metastatic foci in the lungs of nude mice with 95D-ASX4 cells compared with mice with 95D or 95D-pC cells (Fig. 7A and B; P < 0.05).

**Discussion**

For years, chemokines have primarily been thought as instigators of leukocyte trafficking in the immune system (6). Now, fresh data force their role in cancer to be revisited and greatly broadened. There have been ample recent precedents supporting the involvement of chemokine receptors in progression and metastasis of cancer cells (20–23). Phillips et al. (12) and Oonakahara et al. (13) showed that NSCLC express CXCR4 highly, and functional blockade of this receptor by neutralizing antibody leads to the inhibition of metastasis to bone marrow, lymph nodes, or pleural space. Their results strongly suggested the importance of chemokine/chemokine receptor signals in NSCLC metastasis and encouraged us to examine the relationship between the protein expression level of CXCR4 and the clinical features of various NSCLC and evaluated the potential of CXCR4/SDF-1 as therapeutic target in metastatic NSCLC.

We initially proposed that most NSCLC would contain carcinoma cells with various degrees of CXCR4 expression at the protein level and that NSCLC with a high expression of
CXCR4 would have high metastatic potential. To show this, we classified the tumors by CXCR4 expression intensities of immunoreactive cells. NSCLC with high expression of CXCR4 showed clinical metastasis other than those with low expression of CXCR4, which revealed a striking relation between CXCR4 expression and clinical metastasis of NSCLC. This suggested that the movement of NSCLC from primary sites to second sites (regional lymph nodes or distant organ) might be dependent on the expression level of CXCR4. SDF-1, the ligand for CXCR4, has been shown constitutively expressed in a broad range of tissues, especially lymph node, lung, liver, and bone marrow. Therefore, when CXCR4 high tumor cells invaded to regional tissue or metastasize to sentinel lymph nodes, they are liable to be subjected to high concentrations of SDF-1 and exhibit more readily spread than CXCR4 low tumor cells. Previous studies have shown that CXCR4 is expressed in various cancer cell lines (11, 22–28), whereas the immunohistochemical characterization of CXCR4 has been only reported in pancreas cancers and breast cancer (26, 28). In the study by Koshiba et al. (26), they described that CXCR4 was positively stained in the cytoplasm and cell membrane of pancreatic cancer cells but not in normal pancreatic tissue, and interstitial infiltrates showed weak staining. The expression level of CXCR4 did not show a significant relation to the clinicopathologic features of those pancreatic cancers, although they classified the tumors by the intensity of immunoreactive cells.

Both the previous studies and our results implied that blockage of CXCR4/SDF-1 interaction might lead to a novel tool in the prevention and treatment of human metastatic NSCLC. We subsequently evaluated the potential of CXCR4/SDF-1 as a therapeutic target in an in vitro NSCLC culture system. 95C and 95D cells, subcloned from low differentiated human large cell lung carcinoma cell line PLA-801, were of different degrees of metastatic potential. They came from the same cell line and have a similar background, which were an ideal model for studying the mechanism of tumor metastasis (14, 15). In the present study, we have shown that both 95C and 95D cells expressed functional CXCR4. 95D cells pretreated with the same concentration of mouse IgG served as the negative control.
cells expressing higher CXCR4 exhibited a stronger response to SDF-1α and a greater pulmonary metastasis potential in nude mice when inoculated s.c. These implied that CXCR4 expression correlated with the stronger metastatic NSCLC subtype that was associated with poor clinical outcome and further supported the involvement of CXCR4/SDF-1 interaction in the metastasis of human NSCLC.

The metastatic potential of human NSCLC was dependent on several orchestrated events, such as active locomotion, extracellular matrix degradation, and adhesion to vascular endothelial cells. The migration and invasion of a particular cell type could be associated with its metastatic potential could be triggered by chemokine binding to chemokine receptor on the cell surface (17, 18). With up-regulation or down-regulation of CXCR4 expression on NSCLC, the migratory and invasive response to SDF-1α was significantly enhanced or impaired. This provides a possible mechanism of CXCR4/SDF-1 interaction involved in the active locomotion of lung cancer cells. In the process of tumor metastasis, cancer cells of both high and low metastatic potential arrest vessels because of size constraints and that sequence of adhesion and proliferation events determine differences in metastatic potential (29). In this study, a significantly enhanced or reduced adhesive ability to activated vascular endothelial cells was detected in 95C-X4 or 95D-ASX4 cells. Soluble chemokines could be immobilized on the luminal endothelial surface where they exert their proadhesive and migratory effects on blood leukocytes (30, 31). Chemokines might also serve as a “bridge” of linking tumor cells and endothelial cells to facilitate the adhesion and subsequent transmigration. Furthermore, CXCR4high 95D or CXCR4high 95C-X4 cells exhibited high pulmonary metastasis potential, whereas CXCR4low 95D-ASX4 or CXCR4low 95C cells had the impaired metastatic potential, which further supported the idea that modulating CXCR4 expression might inhibit or enhance the metastatic behavior of NSCLC.

In summary, differential expression of CXCR4 in NSCLC was found to correlate with the cell metastatic potential in vitro and in vivo, which suggested that the movement of NSCLC from primary sites to metastatic nodes might be dependent on the level of CXCR4. Disrupting the interaction of SDF-1/CXCR4 by antagonists of CXCR4, such as T140 and its derivates (32), may lead to the design of novel therapeutic tools for the treatment of NSCLC patients.

References

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