Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome

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Background: The expression of CXCR4 has been implicated in metastatic dissemination in different models of breast cancer and melanoma. In the present study, we evaluated CXCR4 expression in non-small-cell lung cancer (NSCLC) and the relationship between CXCR4 expression and the prognosis of stage I disease.

Patients and methods: Using immunohistochemical analysis, we retrospectively analyzed CXCR4 expression in specimens from 61 patients with completely resected pathologically confirmed stage I NSCLC for whom clinical follow-up data were available.

Results: In the present study, we have shown that: CXCR4 is expressed by tumor cells in stage I NSCLC; CXCR4 is located in the nucleus and/or in the cytoplasm of tumor cells; strong nuclear staining was observed in 17 cases (29.8%); patients whose tumors had CXCR4-positive nuclear staining had a significantly longer duration of survival than patients whose tumors had no nuclear expression ($P = 0.039$, log-rank test). Interestingly, the 5-year metastasis rates were 23.5% and 34.1% in patients with CXCR4-positive and CXCR4-negative nuclear expression, respectively ($P = 0.2$).

Conclusion: Strong CXCR4-positive nuclear staining was associated with a significantly better outcome in early-stage NSCLC. The mechanisms underlying this clinically and biologically important finding need to be further explored.

Key words: chemokines, CXCR4, immunohistochemistry, lung cancer, prognostic factor

Introduction

Lung cancer is the leading cause of death from cancer worldwide [1]. Patients presenting with stage I–II disease are usually treated with surgery. Half of these patients however subsequently develop a metastatic relapse that proves fatal in the long run.

Adjuvant chemotherapy has been evaluated in these patients and a meta-analysis suggested that this treatment yields a 5% gain in 5-year survival rates [2]. This meta-analysis has recently been confirmed by a large randomized trial [3]. Neither of these two studies demonstrated an interaction between patient characteristics and the benefit afforded by chemotherapy, which means that a majority of patients with stage I–II NSCLC are going to receive chemotherapy without any benefit in the future. These data clearly indicate a need to determine prognostic factors that could identify patients in whom a metastatic relapse is likely to develop and those in whom this will not occur. Patients in whom adjuvant chemotherapy would really be beneficial could then be selected for such treatment.

Chemokines are low chemotactic factors that regulate the development and migration of various cell types [4]. Over 40 chemokines have been identified. Chemokines can be classified into four groups (CXC, CX3C, CC and C) based on the position of the first two highly conserved cysteines of the amino acid sequence. Chemokine receptors are seven transmembrane G protein-coupled receptors. To date, 19 chemokine receptors have been identified. CXCR4 is a chemokine receptor initially described to be involved in the homing of lymphocytes in inflammatory tissues [5, 6]. The CXCR4 ligand, i.e. CXCL12/SDF1, is expressed in lung, bone and liver, and is known to chemotact lymphocytes in these organs [7]. Based on the lymphocyte homing model, some investigators hypothesized that CXCR4 could be involved in the occurrence of metastasis. CXCR4 has also been shown to be expressed by breast, thyroid, renal and small-cell lung cancer tumor cells, and it appears to be most probably a ubiquitous receptor [8–11]. CXCR4 expression has been implicated in metastatic spread in animal models of breast cancer and melanoma [8, 12]. Indeed, CXCR4 expression on breast or melanoma tumor cell lines is associated with the development of lung metastases, and binding the receptor with an exogenous antibody decreases the formation of lung metastases [8]. It has
recently been shown that CXCR4 mediates the capacity of NSCLC cell lines to metastasize to the lung in mouse models [13]. Based on these data, we hypothesized that CXCR4 expression could be associated with the prognosis in stage I NSCLC.

In the present study, we evaluated CXCR4 expression in NSCLC and the relationship between CXCR4 expression and the prognosis of stage I disease.

## Patients and methods

### Patients

Patients included in the present study were those who presented with a stage I NSCLC treated between 1987 and 1999. The diagnosis was based on histology in all patients. Preoperative staging included a physical examination, blood tests and a CT scan of the chest and abdomen. Bone scintigraphy and a brain CT scan were performed only in case of symptoms. Treatment consisted of surgery alone. None of the patients received postoperative radiotherapy or adjuvant chemotherapy.

### Immunohistochemical staining for CXCR4

Paraffin-embedded, 5-µm-thick tissue sections from all primary tumors were stained for the CXCR4 protein using a primary rabbit polyclonal antibody (Abcam, clone 2074; Cambridge, UK). Slides were deparaffinized through a series of xylene baths. Rehydration was performed through graded alcohols. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 20 min to block endogenous peroxidase activity and incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with primary anti-CXCR4 antibody at dilutions of 1:1000. The sections were then processed using standard avidin–biotin immunohistochemistry according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. CXCR4 expression was evaluated at the cytoplasmic and at the nuclear level. The intensity of staining (1–3) and percentage of positive cells (1 = <10%, 2 = 10–50%, 3 = >50%) were taken into account to define a composite score. Positive nuclear staining was defined as a nuclear score of 6 or 9 [i.e. any slide with >50% of the cells expressing nuclear staining (3) with intermediate or strong intensity (2 or 3)]. Cells were counted in at least three fields (at ×400) in the tumor areas.

Antibody specificity was assessed using a specific blocking peptide (Abcam, ab8126; Cambridge, UK). Incubating the peptide with an equal volume of antibody for 30 min at 37°C usually completely blocks the signal obtained with anti-CXCR4 (ab2074) by immunohistochemistry. Furthermore, specificity was also assessed by performing a western blot analysis with the protein extract of the A-549 NSCLC cell line.

### Statistical analysis

Survival was calculated by the Kaplan–Meier method, and the resulting curves were compared using the log-rank test. Fisher’s exact test and the χ² test were used to analyze the association between two categorical variables. P <0.05 was considered to be statistically significant. Immunohistochemical analysis was performed in a blinded manner with respect to the clinical data concerning the subjects.

### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n = 61)</th>
<th>Weak nuclear staining (n = 44)</th>
<th>Strong nuclear staining (n = 17)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>60.6 (38–84)</td>
<td>60.2 (38–84)</td>
<td>61.7 (40–79)</td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>0.32</td>
</tr>
<tr>
<td>≥50</td>
<td>53</td>
<td>38</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>48 (78.6%)</td>
<td>36 (81.8%)</td>
<td>12 (70.5%)</td>
<td>0.16</td>
</tr>
<tr>
<td>Female</td>
<td>13 (21.4%)</td>
<td>8 (18.2%)</td>
<td>5 (29.5%)</td>
<td></td>
</tr>
<tr>
<td>Tobacco (pack years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>10 (16.4%)</td>
<td>6 (13.6%)</td>
<td>4 (23.5%)</td>
<td>0.18</td>
</tr>
<tr>
<td>≥20</td>
<td>51 (83.6%)</td>
<td>38 (86.4%)</td>
<td>13 (76.5%)</td>
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<tr>
<td>Histologic subtype</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Adenocarcinoma</td>
<td>32 (52.4%)</td>
<td>24 (54.5%)</td>
<td>8 (47%)</td>
<td>0.23</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>22 (36.1%)</td>
<td>16 (36.4%)</td>
<td>6 (35.3%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>7 (11.5%)</td>
<td>4 (9.1%)</td>
<td>3 (17.7%)</td>
<td></td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>28 (45.9%)</td>
<td>22 (50%)</td>
<td>6 (35.3%)</td>
<td>0.13</td>
</tr>
<tr>
<td>T2</td>
<td>33 (54.1%)</td>
<td>22 (50%)</td>
<td>11 (64.7%)</td>
<td></td>
</tr>
<tr>
<td>Metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>19 (31.2%)</td>
<td>15 (34.1%)</td>
<td>4 (23.5%)</td>
<td>0.2</td>
</tr>
<tr>
<td>No</td>
<td>42 (68.2%)</td>
<td>29 (65.9%)</td>
<td>13 (76.5%)</td>
<td></td>
</tr>
</tbody>
</table>
Results

Patient characteristics and survival

Sixty-one patients with pathological stage I NSCLC were included in the present study. Patient characteristics are listed in Table 1. Briefly, the median age was 60.6 years, 13 patients (20%) were female, the adenocarcinoma histologic subtype was present in 32 patients (52.4%) and the tumor was classified as T1 in 28 patients (45.9%). After a median follow-up of 6.3 years, 19 deaths occurred. The 5-year overall survival (OS) rate is 60%. Nineteen patients (31.2%) developed a metastatic relapse. The sites of metastatic relapses were the lung (eight patients), brain (six patients), bone (five patients), liver (four patients), and the adrenal gland (two patients); some patients had multiple metastatic sites. Three patients developed a second primary tumor and one patient died of sepsis.

CXCR4 expression in NSCLC: pattern of expression

CXCR4 was detected in the nucleus and/or in the cytoplasm (Figure 1A and B) of tumor cells, but was not detected in normal bronchial cells. Tumor infiltrating lymphocytes and some fibroblastic cells exhibited positive cytoplasmic staining for CXCR4. Nuclear staining was observed in 17 cases (27.8%), with 14 cases being scored 6 and three cases scored 9. Interestingly, all tumor cells exhibited cytoplasmic staining for CXCR4. The intensity of cytoplasmic staining was usually weak when nuclear staining was present, and intermediate to strong when there was no nuclear staining (there were eight cases with no nuclear staining and a score of 6 or 9 at the cytoplasmic level). The intensity of nuclear staining was always intermediate or strong whenever present. Antibody specificity was assessed using a specific blocking peptide (Abcam, ab8126). Incubating the peptide with an equal volume of antibody for 30 min at 37°C usually completely blocks the signal obtained with anti-CXCR4 (ab2074) by immunohistochemistry. Antibody specificity was also assessed by performing a western blot analysis with the protein extract of the A-549 NSCLC cell line (Figure 2).

There was no significant correlation between CXCR4 expression and age, gender, tobacco consumption or tumor size (Table 1).

CXCR4 expression: correlation with outcome

Positive nuclear staining was associated with better survival compared with negative nuclear staining (5-year OS = 93% versus 52.8%, \( P = 0.036 \)) (Figure 3). The 5-year metastasis rates were 23.5% and 34.1% in patients with positive and negative nuclear expression, respectively \(( P = 0.2 )\). There was no correlation between nuclear staining and the metastatic site, although a trend was seen for lung metastases alone (three out of four versus three out of 15, \( P = 0.06 \)). Interestingly, among the four patients with CXCR4-positive nuclear staining (three with a score of 6 and one with a score of 9) and a metastatic relapse, three are still alive (lung relapse alone). Two of these relapses occurred within 2 years and two after 5 years (thus potentially being second primary tumors). In contrast, 14 out of the 15 patients with a metastatic relapse and CXCR4-negative nuclear staining have died.
Discussion

In the present study, we have shown that CXCR4 is expressed by
tumor cells in stage I NSCLC, CXCR4 is located in the nucleus
and/or in the cytoplasm of tumor cells, and strong nuclear staining
was correlated with better survival.

CXCR4 has been found to be expressed in breast cancer [8],
thyroid cancer [9], rhabdomyosarcoma [14], pancreatic cancer
[15], hepatocellular carcinoma [16] and leukemia [17]. The
present study is the first to report CXCR4 expression in NSCLC,
like that found in small-cell lung cancer [11]. It is noteworthy that
CXCR4 is not found to be expressed in normal lung tissues. Our
data therefore suggest that the expression of CXCR4 by tumor
cells is associated with malignant transformation in the lung. That
CXCR4 may be upregulated during the malignant process is also
suggested in other tumor models. Indeed, in breast cancer,
CXCR4 expression was found in malignant areas, but not in
normal tissue [8]. Similar findings have been reported in thyroid
cancers [9], glioma [18] and pancreatic cancers [15]. Another
argument implicating CXCR4 expression in carcinogenesis is the
finding that the interaction of CXCR4 with SDF1 mediates the
activation of PI3 kinase and Akt and that of cell proliferation [19,
20]. Our data, in line with those previously reported, suggest that
CXCR4 expression by tumor cells is an event that may participate
in malignant transformation.

A striking finding was the presence of CXCR4 in the nucleus in
specimens from 27% of the patients included in our series.
CXCR4 is a serpentine transmembrane protein that mediates
transduction signaling and has been described to be present either
on the cell membrane [20] or in the cytoplasm. To date, only one
study has reported nuclear localization of CXCR4, and this was in
hepatocellular carcinoma [16]. The mechanism mediating the
nuclear expression of CXCR4 has yet to be elucidated.

CXCR4-positive nuclear staining was associated with better
survival in our study. Indeed, the 5-year OS rates were 93% and
52% for patients with strong and weak nuclear staining, respec-
tively. This is the first demonstration that the location of the
expression of a chemokine receptor is associated with outcome.

Other authors have previously reported on the prognostic value
of specific chemokines such as CCR2 (MCP-1) in breast cancer or
CCR7 in gastric cancer [21, 22]. Our finding raises several ques-
tions: Are these clinical data supported by experimental findings
(in vitro or in animal models)? What are the clinical implications
of these data? We have stated previously that CXCR4 is a trans-
membrane protein whose activity is mediated via interaction with
a soluble chemokine (SDF1). It could be speculated that the
nuclear location of CXCR4 inhibits its ability to mediate the
signal provided by SDF1, thereby decreasing cell proliferation
and metastasis. Zeelenberg et al. [23] reported that the retention
of CXCR4 in intracellular compartments (endoplasmic reticulum) of
T-cell hybridoma reduced metastasis and increased the survival of
mice. In another study, Kollet et al. [24] reported that hematopoietic
stem cells exhibiting intracytoplasmic but not transmembrane
CXCR4 were unable to migrate in bone marrow. These studies
highlight the fact that the intracellular retention of CXCR4 is
associated with decreased homing of malignant and normal cells.

It has recently been shown that cisplatin-based chemotherapy
improves the 5-year OS rates of patients presenting with stage I–III
NSCLC [3]. However, the absolute benefit provided by chemother-
apy is modest since 5-year OS rates were, respectively, 45% and
40% for patients treated with chemotherapy and for those who
were not [3]. As no clinical characteristic was correlated with the
benefit afforded by chemotherapy, there is a need to find biomarkers
that could be used to select patients for chemotherapy. In our
study, strong CXCR4-positive nuclear staining was associated with
a 93% 5-year OS rate in patients with stage I NSCLC, com-

![Graph with survival data]

Figure 3. Overall survival according to CXCR4 expression.
pared with a 52% OS rate in patients with weak nuclear staining. This suggests that patients with strong nuclear staining for CXCR4 are not candidates for adjuvant chemotherapy. This finding needs to be confirmed in a larger series since only 61 patients were included in the present study.

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References