ORIGINAL ARTICLE

Expression of CXCR4 and VEGF-C is correlated with lymph node metastasis in non-small cell lung cancer

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Abstract

Background: This study investigated the correlations between CXCR4 and VEGF-C expression and lymph node metastasis in non-small cell lung cancer (NSCLC).

Methods: Tumor specimens, lymph nodes, and normal lung tissues were obtained from 110 NSCLC patients who underwent complete resection. Quantitative reverse transcription-PCR and immunohistochemistry assays were conducted to evaluate messenger RNA (mRNA) and protein expression of CXCR4 and VEGF-C. Logistic regression analysis was performed to determine the independent risk factors for lymph node metastasis in NSCLC.

Results: CXCR4 and VEGF-C mRNA expression were observed in 78 (70.9%) and 64 (58.2%) lung cancer tissues, while CXCR4 and VEGF-C protein expression were observed in 76 (69.9%) and 58 (52.7%) lung cancer tissues, respectively. The expression rates of CXCR4 and VEGF-C mRNA in metastatic lymph nodes were 84.8% and 66.7%, which were higher than that in non-metastatic lymph nodes (27.3% and 18.2%), respectively. Logistic regression analysis revealed that positive expressions of CXCR4 and VEGF-C mRNA were independent risk factors for lymph node metastasis in NSCLC. Furthermore, combined expression of CXCR4 and VEGF-C showed a much higher odds ratio than CXCR4 or VEGF-C expression alone.

Conclusions: CXCR4 and VEGF-C were highly expressed in lung cancer tissues and metastatic lymph nodes. CXCR4 and VEGF-C expression levels were significantly correlated with lymph node metastasis in NSCLC. CXCR4 and VEGF-C might synergically promote lymphatic metastasis in lung cancer and might be a clinical predictor of lymph node metastasis in NSCLC patients.

Introduction

Primary lung cancer is the leading cause of cancer-related death. Although recent advances have been made in therapeutic approaches, such as surgery, chemotherapy, and radiotherapy, the prognosis of lung cancer remains unsatisfactory.[1–3] High incidence of lymphatic metastasis is a chief cause of morbidity and mortality in lung cancer patients, and is closely related to poor prognosis.[4] To improve prognosis, it is essential to clarify the biological characteristics of lymph node metastasis in lung cancer. However, the mechanisms underlying the lymphatic spread of tumor cells in lung cancer are still not fully understood.

Previous studies have indicated that lymph node metastasis in lung cancer is positively correlated with tumor chemotactic migration and lymphangiogenesis.[5] Recent research has revealed that high expression levels of chemokines are related to poor prognosis and chemotherapy tolerance in cancer patients.[6] CXCR4 is a chemokine receptor that plays a critical role in the process of lymphocyte homing to lymphatic vessels and secondary lymphoid organs, including the lymph nodes. It has also been reported that CXCR4 is implicated in the chemotactic migration of cancer cells.[7] VEGF-C is another factor strongly correlated to lymphatic metastasis. VEGF-C binds to VEGFR-3, which could induce lymphangiogenesis, and mediate tumor cell dissemination and the formation of lymph node metastasis.[8–10]

However, studies of the predictive value of CXCR4 in lymph node metastasis of cancer have lead to highly controversial conclusions. It has been reported that CXCR4 protein expression cannot be considered a predictive indicator of lymph node metastasis.[11] Few reports have focused on the relationship between VEGF-C expression and lymph node metastasis in lung cancer. Thus, to clarify the correlation between CXCR4 and VEGF-C expression and lymph node metastasis in lung cancer, CXCR4 and VEGF-C expression was evaluated by PCR and immunohistochemistry in non-small cell lung cancer (NSCLC) patients with and without lymph node metastases.

Methods

Patients

One hundred and ten patients with primary NSCLC who underwent complete lobectomy without any preoperative therapy at the Department of Thoracic Surgery of the Provincial Hospital Affiliated to Shandong University from March 2013 to January 2014 were included in this study. Histological cell typing was conducted according to World Health Organization (WHO) classifications and systematic lymph node dissection was performed according to American Thoracic Society lymph node mapping. Pathologic stage was classified based on the 2009 International Union Against Cancer tumor node metastasis (TNM) classification. Written informed consent was obtained from each patient. The ethical committee of the Provincial Hospital Affiliated to Shandong University approved the study protocol (Ethical Review of Medical Research on Human Beings No. 2015–032).

During surgery, tumor specimens, lymph nodes, and normal lung tissues were obtained and quickly frozen in liquid nitrogen for RNA extraction, or immediately fixed in 10% neutral-buffered formalin for routine pathologic examination. In total, 514 lymph nodes were obtained, among which 157 positive nodes with malignant neoplasms and 90 negative nodes without malignant neoplasms were selected. We also randomly selected 63 normal lung tissues (at a distance of 5 cm from the tumor and confirmed by pathology as normal lung tissues) as the control.

Real-time fluorescence quantitative PCR

Messenger RNA (mRNA) expression of CXCR4 and VEGF-C was analyzed by real-time fluorescence quantitative reverse transcription-PCR (qRT-PCR). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA according to the manufacturer's instructions (Takara, Otsu, Japan). SYBR-Green was used to detect the qRT-PCR products in a LightCycler 480 Real-Time PCR system (Roche

Diagnostics, Indianapolis, IN, USA). The primer sequences of CXCR4 and VEGF-C were as follows: CXCR4: forward primer: 5′-GGCCAAGTTCTTAGTTGCTGTATG-3′, reverse primer: 5′-ACGTTCCACGGGAATGGAG-3′; VEGF-C: forward primer: 5′-AGCACGAGCTACCTCAGCAAGAC-3′, reverse primer: 5′-TTTAGACATGCATCGGCAGGAA-3′

The β-actin gene was amplified as an internal normalization to calculate the relative mRNA expression.

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of lung cancer tissue and lymph nodes were collected. Sections were cut and mounted on poly-L-lysine-coated glass slides. Immunohistochemical staining was performed using the streptavidin peroxidase method. The primary antibodies used in this study were mouse anti-human CXCR4 and VEGF-C monoclonal antibodies with a dilution of 1:200 (ThermoFisher Scientific, Fremont, CA, USA). Negative controls were set by replacing the primary antibody with phosphate buffered saline. Three independent pathologists blinded to the clinical data evaluated all sections. In cases of a discrepancy, the pathologists reached agreement by reanalysis and discussion. The immunostaining results were analyzed using a semi-quantitative scoring system based on the combined evaluation of intensity and proportion of the positively-stained cells. Staining intensity was divided into four grades: 0 = no reactivity, 1 = low, 2 = moderate, and 3 = strong. The proportion of stained tumor cells was also divided into four grades: 0 = 0–5%, 1 = 5–10%, 2 = 11–50% and 3 ≥ 50%. CXCR4 or VEGF-C staining was considered positive when the total score reached 4–9.

Statistical analysis

All data were collected and analyzed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Chi-square or Fisher's exact tests were used to evaluate the differences in CXCR4 and VEGF-C expression between the groups. Logistic regression analysis was performed to determine the relevant risk factors for lymph node metastasis in lung cancer. Differences with P values < 0.05 were considered statistically significant.

Results

Clinical characteristics of the patients

In total, 110 patients with primary NSCLC who underwent complete resection were included in this study. The study population included 78 men and 32 women with an average age of 57 years (range 34–74). Forty-three cases were classified as squamous cell carcinomas, 61 as adenocarcinomas,

Table 1 mRNA expression detected by qRT-PCR

	CXCR4			VEGF-C		
Feature	(+)	(−)	P	(+)	(−)	P
Gender						
Male	54	24	0.65	42	36	0.20
Female	24	8	—	22	10	—
Age						
≤ 60	50	16	0.20	42	24	0.17
> 60	28	16	—	22	22	—
Histology						
Squamous cancer	35	8	0.08*	31	12	**0.04***
Adenocarcinoma	39	22	—	31	30	—
Others	4	2	—	2	4	—
Differentiation						
High and middle	60	28	0.30**	50	38	0.63
Low	18	4	—	14	8	—
TNM						
I-II	32	28	**0.00**	24	36	**0.00**
III-IV	46	4	—	40	10	—
T						
T1–2	42	26	**0.01**	32	36	**0.00**
T3–4	36	6		32	10	
N						
N0	20	24	**0.00**	18	26	**0.00**
N1–2	58	8	—	46	20	—
CXCR4						
(+)	—	—	—	46	32	0.83
(−)	—	—	—	18	14	—
Total	78	32	—	64	46	—

*Squamous/adenocarcinoma.**Fisher's exact test. Bold text indicates that P < 0.05 was statistically significant. mRNA, messenger RNA; qRT, quantitative reverse transcription; TNM, tumor node metastasis.

Table 2 CXCR4 and VEGF-C expression in lung cancer detected by immunohistochemistry

	CXCR4			VEGF-C		
Feature	(+)	(−)	P	(+)	(−)	P
Gender						
Male	52	26	0.50	38	40	0.21
Female	24	8	—	20	12	—
Age						
≤ 60	50	16	0.09	40	26	0.05
> 60	26	18	—	18	26	—
Histology						
Squamous CA	33	10	0.20*	27	16	0.163
Adenocarcinoma	39	22	—	29	32	—
Others	4	2	—	1	2	—
Differentiation						
High and middle	58	30	0.20**	42	46	0.06
Low	18	4	—	16	6	—
TNM						
I–II	30	30	**0.00**	22	38	**0.00**
III–IV	46	4	—	36	14	—
T						
T1–2	40	28	**0.00**	28	40	**0.00**
T3–4	36	6	—	30	12	—
N						
N0	20	24	**0.00**	14	30	**0.00**
N1–2	56	10	—	44	22	—
CXCR4						
(+)	—	—	—	23	15	**0.02**
(−)	—	—	—	6	11	—
Total	76	34	—	58	52	—

*Squamous/adenocarcinoma.**Fisher's exact test. Bold text indicates that P < 0.05 was statistically significant. TNM, tumor node metastasis.

five as adenosquamous carcinomas, and one case was classified as large cell carcinoma. There were 66 patients with N1–2 stage and 44 with N0 stage. The clinicopathological features of the NSCLC patients are summarized in Tables 1 and 2.

CXCR4 expression and its correlation with clinicopathological factors in non-small cell lung cancer (NSCLC)

Messenger RNA expression levels of CXCR4 were detected in lung tumor tissues, lymph nodes, and normal lung tissues. The results are shown in Table 1 and Figure 1. CXCR4 mRNA was highly expressed in 78 lung tumor tissues (70.9%) among the 110 cases. CXCR4 mRNA was detected in 58 (87.9%) out of 66 lung cancer patients with stage N1–2, and 20 (45.5%) out of 44 cases of primary tumors in lung cancer patients with stage N0.

Immunohistochemical staining revealed CXCR4 protein expression in the cytoplasm of lung cancer cells (Table 2 and Fig 2). No positive staining was observed in the adjacent normal lung tissues. In 110 cases of primary tumors,

CXCR4 protein expression was detected in 76 cases (69.1%). CXCR4 protein was detected in 56 (84.8%) out of 66 lung cancer patients with stage N1–2, and 20 (45.5%) out of 44 cases of primary tumors in lung cancer patients with stage N0.

There was no significant correlation in CXCR4 mRNA or protein expression in lung cancer tissues to patients' clinical characteristics, such as age, gender, histology, and differentiation (P > 0.05).

VEGF-C expression and its correlation with clinicopathological factors in NSCLC

Messenger RNA expression levels of VEGF-C were detected in lung tumor tissues, lymph nodes, and normal lung tissues (Fig 1). VEGF-C mRNA was highly expressed in 64 lung tumor tissues (58.2%) among the 110 cases. VEGF-C mRNA was detected in 46 (69.7%) out of 66 lung cancer patients with stage N1–2, and 18 (40.9%) out of 44 lung cancer patients with stage N0. VEGF-C mRNA expression in lung cancer tissues was significantly correlated with histology, stage, tumor size, and lymph node metastasis (P < 0.05).

Figure 1 Quantitative reverse transcription-PCR analysis to detect CXCR4 and VEGF-C expression in non-small cell lung cancer tissue. Relative messenger RNA (mRNA) levels of (**a**) CXCR4 and (**b**) VEGF-C.
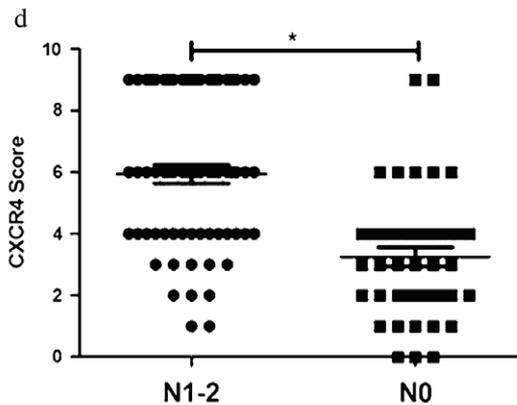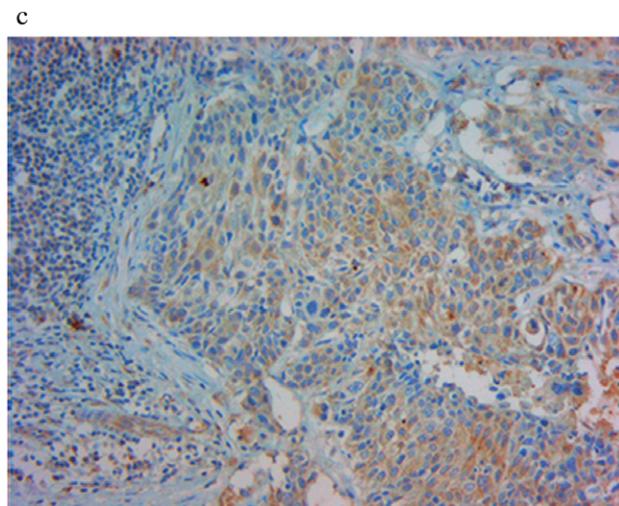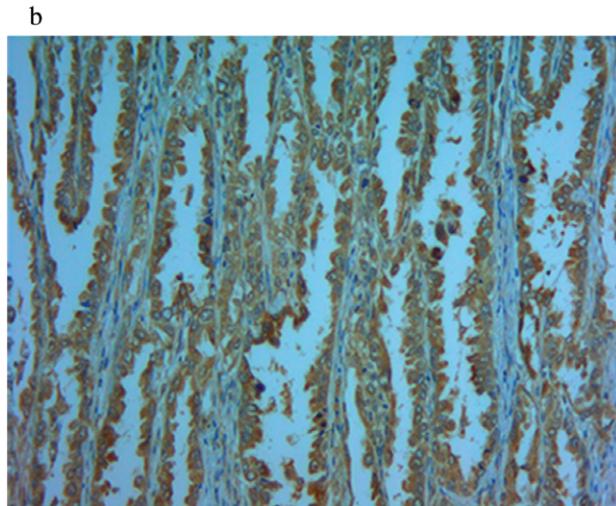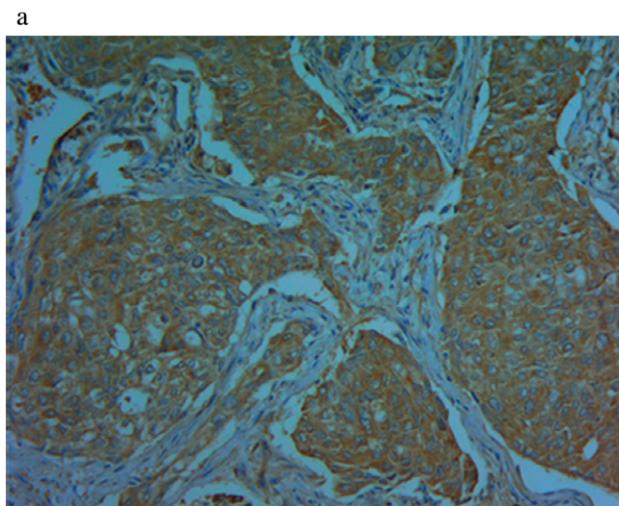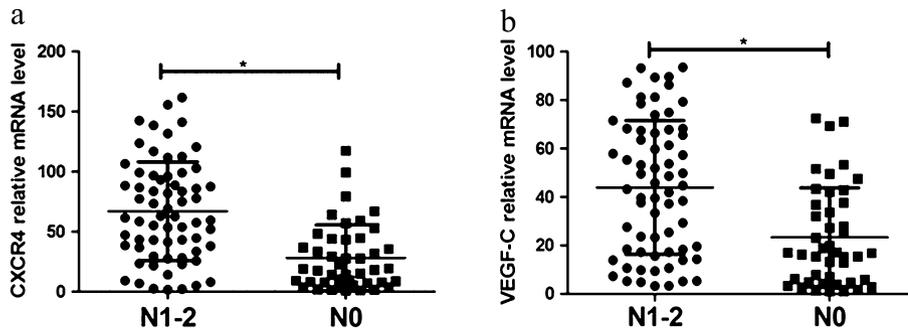

Figure 2 Immunohistochemistry assay of CXCR4 in non-small cell lung cancer tissue and metastatic lymph node tissue. CXCR4 expression in (**a**) squamous cell lung cancer 200×, (**b**) lung adenocarcinoma 200×, and (**c**) metastatic lymph nodes 200×. (**d**) Immunohistochemical scores of CXCR4 were calculated in lymph node metastasis (N1–2) and non-lymph node metastasis groups (N0).

Immunohistochemical staining revealed VEGF-C protein expression. No positive staining was observed in the adjacent normal lung tissues. VEGF-C protein expression was detected in 58 (52.7%) out of 110 cases of primary tumors. VEGF-C protein was detected in 44 (66.7%) out of 66 lung cancer patients with stage N1–2, and in 14 (31.8%) out of 44 lung cancer patients with stage N0. VEGF-C protein expression in lung cancer tissues was significantly
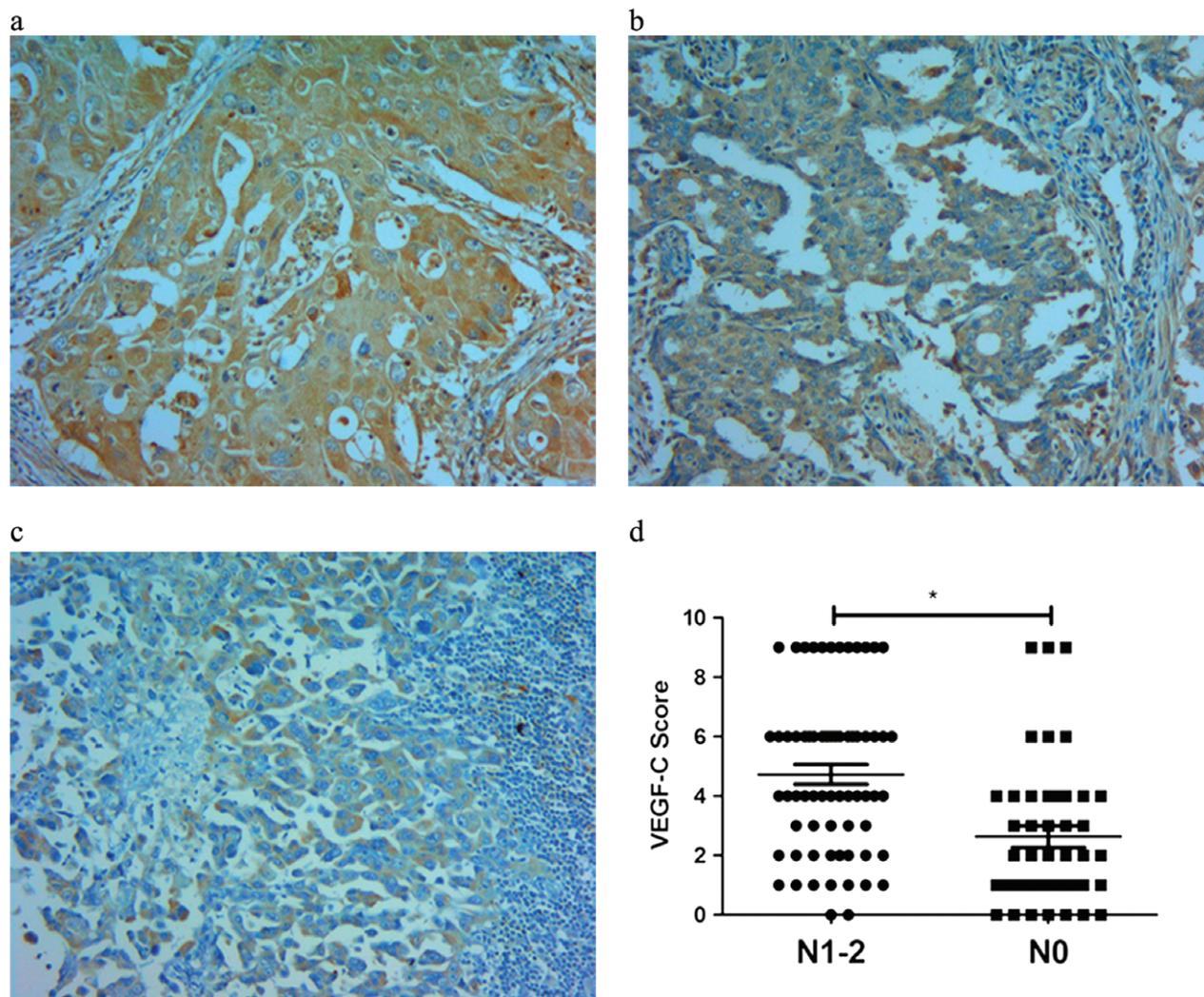
Figure 3 Immunohistochemistry assay of VEGF-C in non-small cell lung cancer tissue and metastatic lymph node tissue. VEGF-C expression in (**a**) squamous cell lung cancer 200×, (**b**) lung adenocarcinoma 200×, and (**c**) metastatic lymph nodes 200×. (**d**) Immunohistochemical scores of VEGF-C were calculated in lymph node metastasis (N1–2) and non-lymph node metastasis groups (N0).

correlated with stage, tumor size, and lymph node metastasis ($P < 0.05$) (Fig 3).

Moreover, both CXCR4 and VEGF-C mRNA were highly expressed in 48 cases (43.6%), while CXCR4 and VEGF-C protein expression was observed in 46 cases (41.8%). Interestingly, we determined that CXCR4 protein expression in lung cancer tissues was correlated with VEGF-C expression ($\chi^2 = 6.00$; $P = 0.022$).

Logistic regression analysis

Multivariate analysis was performed to evaluate CXCR4 and VEGF-C expression as risk factors of lymph node metastasis in lung cancer using the logistic regression model (Table 3). Positive expression of CXCR4 mRNA

(odds ratio [OR] 9.771; $P = 0.000$) and VEGF-C mRNA (OR 5.190; $P = 0.002$) were independent risk factors for lymph node metastasis in lung cancer. No significant

Table 3 Logistic multi-factor regression analysis for CXCR4 and VEGR-C expression

Factor	P	OR	95% CI for OR
Gender	0.885	1.094	0.322–3.718
Age	0.921	1.055	0.367–3.033
Histology	0.196	2.116	0.679–6.597
Differentiation	0.278	2.180	0.533–8.913
T	0.251	0.510	0.161–1.609
CXCR4	**0.000**	9.771	3.212–29.724
VEGF-C	**0.002**	5.190	1.811–14.873

Bold text indicates that $P < 0.05$ was statistically significant. CI, confidence interval; OR, odds ratio.

Table 4 Logistic multi-factor regression analysis for combined positive expression of CXCR4 and VEGF-C

Factor	*P*	OR	95% CI for OR
Gender	0.413	1.651	0.497–5.482
Age	0.955	1.031	0.357–2.977
Histology	0.641	1.311	0.420–4.096
Differentiation	0.428	1.825	0.413–8.068
T	0.203	0.434	0.120–1.569
Both positive	**0.000**	35.545	8.306–152.118

Bold text indicates that *P* < 0.05 was statistically significant. CI, confidence interval; OR, odds ratio.

correlation between lymph node metastasis and gender, age, histology, differentiation, or T status was found. The combined expression of CXCR4 and VEGF-C showed a much higher OR value than CXCR4 or VEGF-C expression alone (OR 35.545; *P* = 0.000) (Table 4).

Discussion

Metastasis is a highly organized, non-random, organ-specific, and multi-step process.[12] Until now, the precise regulatory factors and underlying mechanisms regulating cancer cell migration and invasion into lymph nodes has not been fully understood. Recently, studies have revealed the role of chemokine and lymphangiogenesis in lymph node metastasis.[6,8,12–15] The chemokine system is a family comprising more than 40 chemokines which interact with seven transmembrane G-protein-coupled receptors and is involved in many biologic and pathologic processes, including inflammation, HIV infection, angiogenesis, and tumor growth.[14–16]

The migration and adhesion of tumor cells to target organs are crucial steps in tumor metastasis.[3,17] Recently, several studies have indicated that the immune microenvironment is significantly associated with clinical outcomes in lung cancer.[18,19] Takahashi *et al.* reported that a high neutrophil-lymphocyte ratio (NLR) was associated with more frequent distant metastasis in patients with completely resected NSCLC.[20] Another study indicated that a high CD10+/low CD20+ immune cell infiltration ratio was a significant prognostic factor for lung squamous cell carcinoma. The CXCR4/SDF-1 axis, as the chemokine system, is also related to the inflammatory process. Tumor cells can utilize and manipulate the chemokine system in a manner that benefits both local tumor growth and distant dissemination.[21,22] Cancer cells can be attracted and activated by chemokines through chemokine receptors on their cell membranes.[23] CXCR4 is widely expressed in more than 23 different types of malignant tumors including kidney, lung, brain, prostate, breast, pancreas, ovarian, and melanomas. CXCR4 could induce multiple oncogenic alternations and promote survival, proliferation, invasion, metastasis, and therapeutic resistance of tumor cells

through integrating with its ligand, CXCL12.[13,24] High levels of CXCR4 expression were found in breast cancer cells and is thought to indicate the metastatic destination of tumor cells.[15] Recently, Bertolini *et al.* reported that compared to NSCLC cells with low levels of CXCR4 expression, NSCLC cells with high CXCR4 expression levels demonstrated a capacity for high self-renewal and chemotherapeutic resistance.[25] It is well known that tumor cell migration and metastasis processes share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors.[26] Therefore, chemokine-mediated mechanisms might also be involved in the process of lymph node metastasis of cancer cells, just as the regulation of chemokines in lymphocyte trafficking. However, the predicted effect of CXCR4 on lymph node metastasis in NSCLC patients has not yet been clarified in previous studies.

Our study demonstrated that CXCR4 was highly expressed in lung cancer tissues and positive metastatic lymph nodes. Using multivariate analysis, we demonstrated that CXCR4 was an independent risk factor for lymph node metastasis in NSCLC.

On the other hand, lymph node metastasis has been related to increased intratumor and/or peritumor lymphangiogenesis. VEGF-C is an important regulator of lymphangiogenesis and an important mediator of tumor metastasis toward sentinel lymph nodes.[27] Most likely, lymphatic metastasis involves both invasion toward surrounding lymphatic vessels and either induction of new lymphatic sprouts into the tumor or expansion of peritumoral lymphatic vessels.[28,29] Issa *et al.* reported that VEGF-C could enhance tumor cell chemoinvasion toward lymphatics binding with autologous ligation of tumor VEGFR-3, which is expressed in low levels when tumor cells are cultured in a three-dimensional environment, leading to an increase in the proteolytic activity of the tumor cells and thereby enhancing their migration potential.[29] Using qRT-PCR and immunohistochemistry assays, we confirmed that VEGF-C was also highly expressed in lung cancer tissues and metastatic lymph nodes, and VEGF-C was an independent risk factor for lymph node metastasis in NSCLC. The results suggested VEGF-C might be involved in the molecular signals for lymphangiogenesis in NSCLC. The specific molecular pathway signals for lymphangiogenesis in lung cancer should be pursued in future analyses of tumor lymphatic metastasis.

Interestingly, our study indicated that CXCR4 protein expression in lung cancer tissues was correlated with VEGF-C expression. We also determined that combined CXCR4 and VEGF-C expression showed a much higher OR value than CXCR4 or VEGF-C expression alone. Yasuoka *et al.*'s study showed similar results in that the combination of cytoplasmic CXCR4 and VEGF-C

expression was correlated with lymph node metastasis more strongly than cytoplasmic CXCR4 or VEGF-C expression alone.[12] Earlier studies have shown that VEGF-C stimulation could induce CXCR4 upregulation in lymphangiogenic endothelial cells.[13] Taken together, these results provide evidence that marked expression of CXCR4 and VEGF-C may play an important role in tumor growth, angiogenesis, and metastasis. We speculated that CXCR4 and VEGF-C expression could synergistically promote tumor cell invasion to the lymphatics, possibly because CXCL12 binding to CXCR4 activates PI3K. The activated PI3K then rapidly generates phosphatidylinositol (3,4,5)-trisphosphate and initiates AKT activation.[15] Activated AKT stimulates NF-κB activation and eventually upregulates COX-2 expression.[30] COX-2 is reportedly increased in VEGF-C mRNA transcription.[31] Our study results suggested that CXCR4 and VEGF-C might be used as potential molecular targets for the diagnosis of lymph node metastasis in lung cancer. Attention should be paid to detecting the positive expression of CXCR4 and VEGF-C combined.

There are some limitations to this study. The patient sample was derived from one institution in the same area, which may limit the generalization of our results. The small sample size and the fact that this is a primary study are also limitations. Further research is needed to validate the clinical significance of CXCR4 and VEGF-C.

In summary, CXCR4 and VEGF-C were highly expressed in metastatic lymph nodes and cancer tissues in NSCLC. CXCR4 and VEGF-C expression were significantly correlated with lymph node metastasis in NSCLC. CXCR4 and VEGF-C expression, particularly in combination, might promote lymphatic metastasis in lung cancer and may potentially be a clinical predictor of lymph node metastasis in NSCLC patients.

Acknowledgments

Disclosure

No authors report any conflict of interest.

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