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# Significance of CXCR4, phosphorylated STAT3 and VEGF-A expression in resected non-small cell lung cancer

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**Abstract.** C-X-C chemokine receptor type 4 (CXCR4) plays an important role in determining the metastatic potential of non-small cell lung cancer. In order to elucidate the effect and mechanism of CXCR4 in tumor angiogenesis we evaluated the clinical significance of CXCR4, phosphorylated signal transducer and activator of transcription 3 (P-STAT3), and vascular endothelial growth factor (VEGF) expression in patients with completely resected non-small cell lung cancer (NSCLC). A total of 208 cases of resected NSCLC were collected, and expression of CXCR4, P-STAT3 and VEGF-A in tumor tissue was investigated using immunohistochemistry (IHC). We reviewed the patient clinical records to determine the association of the expression of these proteins with the clinical course of the disease. Expression of CXCR4, P-STAT3 and VEGF-A was detected in 56.3, 46.2 and 51.9% of the samples, respectively. We observed co-expression between CXCR4, P-STAT3 and VEGF-A. Using multivariate analysis, the expression levels of CXCR4 and VEGF-A were identified as independent prognostic factors that affected overall survival. In conclusion, the results of this study suggest that CXCR4, P-STAT3 and VEGF-A expression may play a role in tumor progression and angiogenesis of NSCLC. However, further studies are needed to uncover the detailed mechanism that underlies the role of these proteins in NSCLC.

## Introduction

Non-small cell lung cancer (NSCLC) is the most common malignancy in northern China, despite its declining incidence. The estimated overall 5-year survival rate is only 16% (1). A more complete understanding of the molecular mechanism of lung cancer will aid in the development of new treatment modalities, diagnostic technologies and preventive approaches. Thus, increased numbers of molecular markers need to be investigated in order to clarify the features of NSCLC and to ensure effective treatment.

Recent research indicates that the expression of chemokine receptors plays an important role in determining the metastatic potential of tumor cells (2). C-X-C chemokine receptor type 4 (CXCR4) is functionally expressed on the cell surface of various types of cancer cells and plays a role in the cell proliferation and migration of these cells (3). CXCR4 belongs to the superfamily of seven transmembrane domain heterotrimeric G protein-coupled receptors that are physiologically involved in the migration of various hematopoietic cells to home-specific anatomical sites through local interaction with their specific ligands. CXCR4 is overexpressed in colorectal cancer (4), hepatocellular carcinoma (5), ovarian cancer (6), and renal cell carcinoma (7), and is associated with chemotaxis, invasion, angiogenesis and cell proliferation.

Signal transducer and activator of transcription 3 (STAT3) is a key pathway that regulates metastasis in human cancer cells (8), and persistent activation of STAT3 may lead to oncogenesis by promoting tumor angiogenesis, cell proliferation, and resistance to apoptosis (9). In invasive breast cancer tissue, elevated levels of STAT3 phosphorylation were significantly associated with increased expression of downstream targets of STAT3, including inducers of tumor angiogenesis [vascular endothelial growth factor (VEGF), metalloproteinase (MMP)-2, MMP-10 and cyclooxygenase (COX)-2] (10). Tumor growth and development is a complex, multistep process; one essential step for tumor growth is angiogenesis, which plays a critical role in tumor invasion and metastasis (11). VEGF is believed to be the principal growth stimulatory factor for tumor-related angiogenesis. Constitutive activation of STAT3 is able to up-regulate VEGF-A expression in human pancreatic and colorectal cancer cells (12,13).

Therefore, the clinical significance and possible prognostic value of CXCR4, P-STAT3 and VEGF-A expression in patients with completely resected NSCLC was evaluated in the present study to determine the effects of CXCR4 in tumor angiogenesis and metastasis.

## Materials and methods

*Patients and samples*. A total of 208 cases with pathologically confirmed NSCLC were involved in this study. The patients

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*Key words:* C-X-C chemokine receptor type 4, phosphorylated signal transducer and activator of transcription 3, vascular endothelial growth factor, immunohistochemistry, prognosis, non-small cell lung cancer

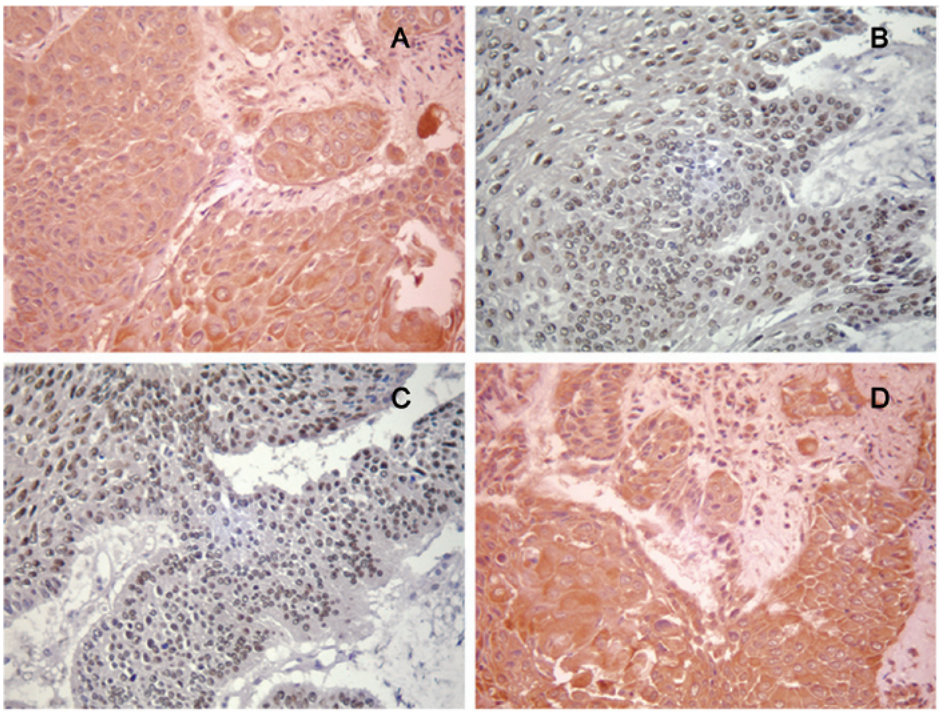


Figure 1. Immunohistochemical expression of (A and B) CXCR4, (C) P-STAT3 and (D) VEGF-A protein in NSCLC tissues (SP method, x200). (A) Immunoreactivity was observed in the cytoplasm of the malignant cells. Brown granules in the cytoplasm of the NSCLC cells indicate CXCR4 protein. (B) Nuclear staining of CXCR4 protein in the NSCLC tissues, and (C) nuclear staining of P-STAT3 protein in the NSCLC tissues. (D) Brown granules in the cytoplasm of the NSCLC cells indicate VEGF-A expression.

underwent potentially curative tumor resection at the Tumor Hospital of Harbin Medical University from 2002 to 2004. The patients received neither chemotherapy nor radiation therapy prior to surgery. Routinely processed formalin-fixed, paraffin-embedded blocks containing principal tumors were selected for the study. Serial sections (4 $\mu$m) were prepared from the cut surface of the blocks at the maximum cross-sectional location of the tumor. Informed consent was obtained from all patients. The study was approved by the Ethics Committee of the Harbin Medical University.

*Immunohistochemistry*. Immunohistochemical staining for the CXCR4, P-STAT3 and VEGF-A antigen was performed using the standard streptavidin-peroxidase biotin technique (SP technique) with a SP kit (Zhongshan Co., Beijing, China). Paraffin sections (4 $\mu$m) were deparaffinized in xylene and then rehydated through graded alcohol. Hydrated autoclave pre-treatment was carried out by boiling for 5 min in citrate buffer (10 mM, pH 6.0). After endogenous peroxidase was quenched in 3% hydrogen peroxide and blocked for 10 min, the sections were incubated overnight at 4˚C with antibodies against CXCR4 (R&D Systems) at a 1:200 dilution, P-STAT3 (Cell Signalling Technology) at a 1:150 dilution and VEGF-A (Neomarkers) at a 1:200 dilution. Biotinylated immunoglobulin and streptavidin conjugated to peroxidase were then added. Finally, 3,3'-diaminobenzidine was added for color development, and hematoxylin was used for counterstaining. Negative control slides processed without the primary antibody were included for each staining. The mean percentage of positive tumor cells was determined in at least five areas at x200 magnification. All slides were evaluated by experienced pathologists who reviewed the slides together and reached a consensus. Positive expression for P-STAT3 was defined as >25% nuclear staining with greater than moderate staining intensity of tumor cells. The staining of both CXCR4 and VEGF-A was mainly localized in the cytoplasm. CXCR4 and VEGF were scaled as follows: 0, <5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; and 4, >75% positively stained cells. The intensity of immunostaining was scored as follows: 0, none; 1+, weak; 2+, moderate; 3+, intense. The scores for the percentage of positive tumor cells and staining intensity were multiplied together to achieve a weighted score for each case. Cases with weighted scores 0 or 1 were defined as negative; cases with scores ≥2 were defined as positive.

*Statistical analysis*. All data were analyzed by statistical software (SPSS 13.0 for Windows; SPSS, Inc.). The association between CXCR4, P-STAT3 and VEGF-A expression and clinicopathological parameters was analyzed using the $\chi^2$ test. Correlations among the levels of CXCR4, P-STAT3 and VEGF-A protein in NSCLC tissues were determined using Pearson's correlation coefficient (r) analysis. The survival curves were plotted according to the Kaplan-Meier method and validated by the log-rank test. Univariate and multivariate regression analyses were performed using the Cox proportional hazards regression model to analyze the independent factors related to prognosis. For all of the tests, $P<0.05$ was considered to be statistically significant.

## Results

*Patient characteristics*. Data from a total of 208 patients, 128 male and 80 female, were analyzed. The clinicopatho-

Table I. Correlation between expression of CXCR4, P-STAT3 and VEGF-A and clinicopathological features.

| Features | All patients (n=208) | CXCR4 | | | P-STAT3 | | | VEGF-A | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Negative (n=91) | Positive (n=117) | P-value | Negative (n=112) | Positive (n=96) | P-value | Negative (n=100) | Positive (n=108) | P-value |
| Age (years) | | | | | | | | | | |
| <60 | 89 | 37 | 52 | 0.584 | 51 | 38 | 0.387 | 48 | 41 | 0.144 |
| ≥60 | 119 | 54 | 65 | | 61 | 58 | | 52 | 67 | |
| Gender | | | | | | | | | | |
| Male | 128 | 54 | 74 | 0.566 | 67 | 61 | 0.582 | 59 | 69 | 0.469 |
| Female | 80 | 37 | 43 | | 45 | 35 | | 41 | 39 | |
| Smoking | | | | | | | | | | |
| Never | 106 | 47 | 59 | 0.859 | 62 | 44 | 0.320 | 53 | 53 | 0.360 |
| Former | 81 | 36 | 45 | | 41 | 40 | | 40 | 41 | |
| Current | 21 | 8 | 13 | | 9 | 12 | | 7 | 14 | |
| Stage | | | | | | | | | | |
| I | 88 | 54 | 34 | 0.000 | 52 | 36 | 0.231 | 52 | 36 | 0.004 |
| II | 70 | 32 | 38 | | 38 | 32 | | 33 | 37 | |
| III | 50 | 5 | 45 | | 22 | 28 | | 15 | 35 | |
| Tumor classification | | | | | | | | | | |
| T1 | 83 | 49 | 34 | 0.000 | 53 | 30 | 0.090 | 48 | 35 | 0.002 |
| T2 | 73 | 31 | 42 | | 37 | 36 | | 39 | 34 | |
| T3 | 41 | 8 | 33 | | 17 | 24 | | 10 | 31 | |
| T4 | 11 | 3 | 8 | | 5 | 6 | | 3 | 8 | |
| Lymph node status | | | | | | | | | | |
| N0 | 66 | 38 | 28 | 0.011 | 44 | 22 | 0.016 | 43 | 23 | 0.003 |
| N1 | 101 | 41 | 60 | | 52 | 49 | | 39 | 62 | |
| N2 | 41 | 12 | 29 | | 16 | 25 | | 18 | 23 | |
| Tumor size | | | | | | | | | | |
| <2 cm | 71 | 43 | 28 | 0.001 | 37 | 34 | 0.935 | 37 | 34 | 0.462 |
| 2-5 cm | 113 | 42 | 71 | | 62 | 51 | | 54 | 59 | |
| >5 cm | 24 | 6 | 18 | | 13 | 11 | | 9 | 15 | |
| Histology | | | | | | | | | | |
| Squamous-cell carcinoma | 106 | 50 | 56 | 0.241 | 54 | 52 | 0.689 | 49 | 57 | 0.714 |
| Adenocarcinoma | 90 | 34 | 56 | | 51 | 39 | | 44 | 46 | |
| Large-cell carcinoma | 12 | 7 | 5 | | 7 | 5 | | 7 | 5 | |
| Pathological stage | | | | | | | | | | |
| G1 | 41 | 20 | 21 | 0.449 | 27 | 14 | 0.198 | 23 | 18 | 0.518 |
| G2 | 91 | 42 | 49 | | 48 | 43 | | 42 | 49 | |
| G3 | 76 | 29 | 47 | | 37 | 39 | | 35 | 41 | |

logic features are summarized in Table I. The mean age of the patients was 59.8 years (range, 35-76). There were 106 patients who had never been smokers (50.1%). Pathological tumor stage determined according to the American Joint Committee on Cancer classification included 88 (42.3%) stage I, 70 (33.7%) stage II and 50 (24%) stage III cases. Of the total cases, 106 tumors were squamous, 90 were adenocarcinoma and 12 were large-cell carcinomas. Among the patients with stage III disease, 41 (82%) had lymph node-positive (N2) disease, and the remaining patients had T4 or T3 tumors. The median follow-up was 67 months (range, 1-78.2), the median disease-free survival was 32.6 months (95% confidence interval, 12-53.2 months), and the median overall survival was 46.2 months. There were 76 patients who had died by the time of the current analysis, with the most common cause of death being disease progression (57 patients).

*Expression of CXCR4, P-STAT3 and VEGF in NSCLC*. The staining of CXCR4 and VEGF-A was observed predominantly in the cytoplasm of the tumor cells, whereas staining for P-STAT3 appeared in the tumor cell nucleus. Representative immunohistochemical staining of CXCR4, P-STAT3 and

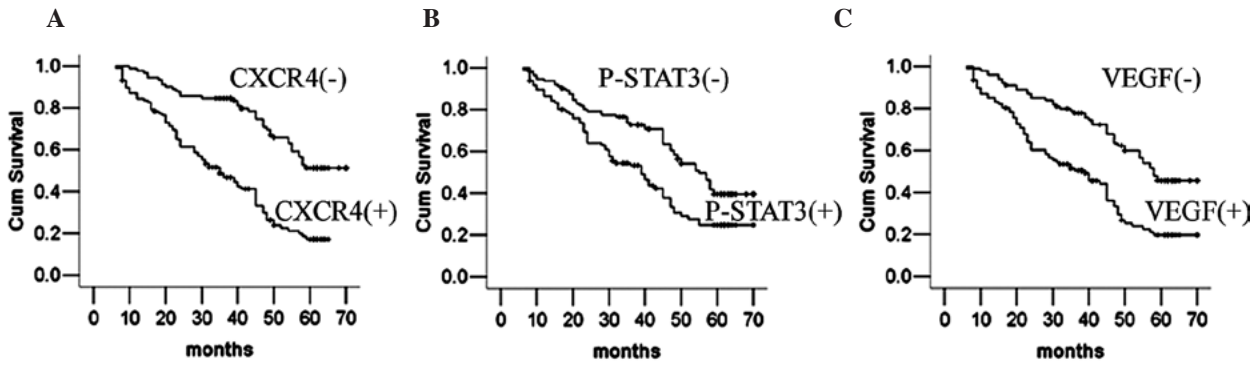


Figure 2. Overall survival curves for patients with resected NSCLC according to the expression levels of CXCR4, P-STAT3 and VEGF-A. (A) The overall survival time of patients with CXCR4-positive cancers was significantly lower compared with the survival time of patients with CXCR4-negative cancers (P<0.05). (B) The overall survival time of patients with P-STAT3-positive cancers was significantly lower compared with the survival time of patients with P-STAT3-negative cancers (P<0.05). (C) The overall survival time of patients with VEGF-A-positive cancers was significantly lower compared with the survival time of patients with VEGF-A-negative cancers (P<0.05).

VEGF-A are provided in Fig. 1. Expression of CXCR4, P-STAT3 and VEGF-A was detected in 117 (56.3%) samples, 96 (46.2%) samples, and 108 (51.9%) samples, respectively. A correlation was noted between P-STAT3 and CXCR4 (r=0.136, P=0.050), P-STAT3 and VEGF-A (r=0.231, P=0.001) and CXCR4 and VEGF-A (r=0.165, P=0.017) (Table II).

*Relationship between protein expression and clinical parameters*. The relationship between CXCR4, P-STAT3 and VEGF-A expression and clinicopathological features of NSCLC is shown in Table I. Statistical analyses were performed to examine the relationship between the expression of CXCR4 and the clinicopathological features of NSCLC. CXCR4 was found to be significantly correlated with tumor classification, lymph node metastasis, stage and tumor size (P<0.05). The expression of P-STAT3 was significantly associated with lymph node metastasis (P<0.05). Moreover, a weak association was found between P-STAT3 and tumor classification (P=0.09). The expression of VEGF-A was significantly correlated with stage, tumor classification and lymph node metastasis (P<0.05).

*Overall survival for patients with resected NSCLC according to expression levels of CXCR4, P-STAT3 and VEGF-A*. The overall survival time of patients with CXCR4-, P-STAT3- and VEGF-A-positive cancers was significantly lower compared with the survival time of patients with CXCR4-, P-STAT3- and VEGF-A-negative cancers (all P<0.05) (Fig. 2).

*Univariate and multivariate analyses of prognostic factors for survival of the NSCLC patients*. Univariate analysis showed that smoking, tumor size, lymph node status, stage, tumor classification, pathological stage, CXCR4, P-STAT3 and VEGF expression were significantly correlated with overall patient survival. We carried out multivariate survival analysis as described in statistical analysis. The findings further revealed that tumor size, lymph node metastasis, tumor classification, stage, CXCR4 and VEGF expression were identified as independent predictive factors (Table III).

## Discussion

Chemokines, produced by cancer-associated fibroblasts, a component of stromal cells, influence the metastatic potential and site-specific dissemination of cancer cells (14). Endogenous CXCR4 expression on carcinoma cells is known to correlate with a poor prognosis for several carcinoma types (15-17). The knockdown of CXCR4 expression by small interfering RNA in breast carcinoma cells decreases cell invasion and adhesion *in vitro* and abrogates tumor growth *in vivo* (18).

Table II. Correlations between CXCR4, P-STAT3 and VEGF-A expression in the NSCLC tissues.

| | P-STAT3 | | | | VEGF-A | | | |
|---|---|---|---|---|---|---|---|---|
| | Negative expression | Positive expression | r | P-value | Negative expression | Positive expression | r | P-value |
| CXCR4 | | | | | | | | |
| Negative expression | 56 | 35 | 0.136 | 0.050 | 54 | 37 | 0.165 | 0.017 |
| Positive expression | 56 | 61 | | | 50 | 67 | | |
| VEGF | | | | | | | | |
| Negative expression | 68 | 36 | 0.231 | 0.001 | | | | |
| Positive expression | 44 | 60 | | | | | | |

Table III. Univariate and multivariate analyses of prognostic factors for survival of the NSCLC patients.

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| Variables | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Age (<60 vs. ≥60 years) | 1.151 | 0.807-1.642 | 0.438 | 1.162 | 0.802-1.683 | 0.427 |
| Tumor size (<2 vs. ≥2 cm) | 2.076 | 1.386-3.110 | 0.000 | 1.638 | 1.074-2.498 | 0.022 |
| Smoking (never vs. former, current) | 1.497 | 1.055-2.125 | 0.024 | 1.073 | 0.748-1.537 | 0.703 |
| Lymph node status (N0 vs. N1, N2) | 3.782 | 2.474-5.781 | 0.000 | 2.341 | 1.684-3.255 | 0.000 |
| Stage (I vs. II, III) | 3.217 | 2.368-4.372 | 0.000 | 2.275 | 1.636-3.162 | 0.000 |
| Tumor classification (T1, T2, vs. T4, T3) | 3.572 | 2.463-5.180 | 0.000 | 1.804 | 1.172-2.778 | 0.007 |
| Pathological stage (G1 vs. G2, G3) | 1.832 | 1.146-2.930 | 0.011 | 1.397 | 0.865-2.259 | 0.172 |
| CXCR4 (negative vs. positive) | 3.006 | 2.049-4.412 | 0.000 | 2.070 | 1.365-3.140 | 0.001 |
| Phosphorylated STAT3 (negative vs. positive) | 1.768 | 1.240-2.520 | 0.002 | 1.290 | 0.886-1.877 | 0.184 |
| VEGF (negative vs. positive) | 2.330 | 1.620-3.353 | 0.000 | 1.751 | 1.194-2.567 | 0.004 |

HR, hazard ratio; CI, confidence interval.

In small-cell lung cancer (SCLC) cells, CXCR4 antagonists such as plerixafor (AMD3100) and T140 analogues (TN14003/BKT140) disrupt CXCR4-mediated SCLC cell adhesion to stromal cells, thereby sensitizing SCLC cells to cytotoxic drugs, such as etoposide, and antagonizing cell adhesion-mediated drug resistance (19). These findings suggest that CXCR4 may be a potent oncogenic molecule that plays an important role in NSCLC.

Our study was comprised of a sufficiently large number of participants to demonstrate that the CXCR4 protein was expressed with great frequency (56.3%) in 208 NSCLC tissue samples. Furthermore, CXCR4 exhibited a significant positive correlation with tumor classification, lymph node metastasis, stage and tumor size. CXCR4 expression was significantly correlated with P-STAT3 expression, and it was an independent prognostic factor for survival. The results strongly indicate that CXCR4 has the potential to be utilized as a novel biomarker to identify the progression of NSCLC and predict high-risk patients. It should be noted that CXCR4 staining in our series was primarily located in the cytoplasm. However, in six cases it was found exclusively in the nucleus. This phenomenon has also been described by Na *et al* (20), and may represent a functional status of the receptor; however, we cannot reach any conclusion concerning the different localization as there was not a sufficient number of examined events.

STAT3 is constitutively activated by numerous cytokines, growth factors and oncogenic proteins in many types of human cancers, and it participates in the regulation of malignant processes (9,21,22); moreover, it can directly or indirectly regulate genes related to cell proliferation, metastasis and survival (23,24). STAT3 is a key pathway that regulates survival in human NSCLC (25). In glioblastoma stem cells (GSCs), knockdown of STAT3 induces apoptosis and significantly reduces the expression of Bcl-2 and cyclin-D, suggesting that STAT3 is an important target for human GSCs (26). STAT3 is important in the metastatic process, and blockage of activated STAT3 significantly suppresses MMP-2 expression, and brain and lung tumor cell metastasis (27). In the current study 46.2% of the patients expressed P-STAT3, consistent with previous studies (28,29). We found a significant association between P-STAT3 and lymph node status (P=0.016), and a weak association between P-STAT3 and tumor classification (P=0.090); this supports the finding that activated STAT3 contributes to the invasion of NSCLC cells. Therefore, activation of STAT3 may play an important role in tumor invasion and nodal metastasis in NSCLC. Moreover, P-STAT3 expression significantly correlated with VEGF expression, but was not identified as an independent prognostic factor for overall survival in the present study.

Angiogenesis, the formation of new tumor-feeding blood vessels from preexisting vasculature, is critical for the development and subsequent growth of human tumors and is a prerequisite for metastasis. VEGF is considered to be the principal vascular growth factor prompting tumor angiogenesis. The expression of VEGF is associated with poor prognosis and increased resistance to therapeutic intervention in several types of neoplasms (11,30). Our study revealed that the overexpression of VEGF-A as determined in the NSCLC tumor tissue samples was significantly correlated with lymph node metastasis, stage and tumor classification. Co-expression was noted between VEGF-A and CXCR4 expression, which is in accordance with a study by Oda *et al* where similar co-expression was reported (17). In the present study, the overexpression of VEGF-A was a poor independent prognostic factor for overall survival.

In the present study, co-expression was found between CXCR4 and P-STAT3, P-STAT3 and VEGF-A, and finally CXCR4 and VEGF-A. CXCR4 expression was observed in 56.3% of the NSCLC samples and was correlated with tumor classification, lymph node metastasis, stage and tumor size. Furthermore, 46.2% of the patients expressed P-STAT3, and a significant association was found between P-STAT3 and lymph node status and a weak association between P-STAT3 and tumor classification. Overexpression of VEGF-A in the NSCLC tumor tissues was significantly correlated with lymph node metastasis, stage and tumor classification. The present study suggests that these proteins may contribute to tumor progression and angiogenesis, and the results also indicate that

CXCR4 may promote metastasis through the STAT3 signaling pathway. Further studies are needed in order to reveal the detailed mechanism that underlies the role that these proteins play in NSCLC.

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