

Association between CXCL16/CXCR6 expression and the clinicopathological features of patients with non-small cell lung cancer

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Abstract. Lung cancer is a major cause of morbidity and mortality worldwide, therefore identifying biomarkers for the early detection, grading or postoperative follow-up of lung cancer is of clinical significance. In the present study, expression of lung tissue (t)-CXCL16 and t-CXCR6 was examined in 58 patients with non-small cell lung cancer (NSCLC) using immunohistochemical staining, and serum (s)-CXCL16 levels were detected in 58 patients with NSCLC and in 32 normal volunteers using an ELISA. A follow-up was performed every 4 months between January 2014 and January 2015. Compared with the normal volunteers, the s-CXCL16 concentration in patients with NSCLC significantly increased (329.47 ± 135.38 vs. 572.82 ± 116.05 pg/ml, respectively; $P < 0.001$). When grouped according to TNM stage, the expression of t-CXCL16 (60 vs. 85.71%; $P = 0.029$), t-CXCR6 (53.33 vs. 78.57%; $P = 0.043$) and s-CXCL16 (26.67 vs. 57.14%, $P = 0.019$) in the stage I-II subgroup was significantly lower compared with that of the stage III-IV subgroup. The positive expression rate of t-CXCL16 (91.18%) and t-CXCR6 (79.41%) in the lymph node metastasis subgroup was significantly higher compared with that of the corresponding non-lymph node metastasis subgroup (50 and 45.83%, respectively; $P < 0.01$). Additionally, the positive

expression rate of t-CXCL16 in the smoking subgroup was 100%, which was significantly higher compared with that of the non-smoking subgroup (23.81%) ($P < 0.001$). The follow-up and mortality rates were 100% (58/58) and 13.79% (8/58), respectively. Within the time period of the present study, the survival time was 4-18 months, and the mean survival time was 16.6 months. In conclusion, the expression of t-CXCL16 and t-CXCR6 is positively correlated with the TNM stage and lymph node metastasis in patients with NSCLC. Additionally, there was a significant increase in s-CXCL16 levels in patients with NSCLC, suggesting that CXCL16 could be used as a supplementary biomarker for the early detection of NSCLC.

Introduction

Lung cancer is a major cause of morbidity and mortality worldwide (1,2). Based on its cellular characteristics, lung cancer is divided into two major types as follows: Non-small cell lung cancer (NSCLC) and small cell lung cancer. NSCLC accounts for >80% of lung cancer diagnoses (3). Although great advancements have been made in non-invasive surgery, chemotherapy, radiotherapy and immunotherapy to treat human cancer, the 5-year survival rate for patients with advanced NSCLC is only 15% (3). Tumor metastasis is one of the primary factors that determines the prognosis, quality of life and survival rate of patients. Therefore, identifying the molecules and signaling pathways associated with cancer metastasis is of clinical significance and may aid in improving the prognosis of patients with NSCLC.

Previous studies have demonstrated that malignancies express a number of chemokines (CKs) and CK receptors (CKRs), suggesting a role for CK/CKR signaling networks in tumor development and progression (4-8). Among these CKs and CKRs, the C-X-C motif CK16 (CXCL16)-CXCR6 receptor type 6 (CXCR6) signaling axis has been highlighted due to its distinctive features. CXCL16 can exist in a transmembrane (t) and soluble (s) form, and CXCR6 is its sole receptor (9-11). Aside from the roles that CXCL16 and CXCR6 serve in normal biological processes, CXCL16 and CXCR6 are also aberrantly expressed in numerous types of human cancer, including prostate, breast, pancreatic, colorectal and bladder cancer, and in

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renal cell carcinoma and nasopharyngeal carcinoma (12-21). The interaction between CXCL16 and CXCR6 is associated with the growth, survival, migration, invasion, angiogenesis and the activation of multiple intracellular signaling pathways in malignant cells (15-21), suggesting that the CXCL16-CXCR6 interaction may serve an important role in tumorigenesis and metastasis.

A previous study confirmed the expression of CXCL16 and CXCR6 in human primary lung cancer tissues, and demonstrated that the activation of the CXCL16-CXCR6 signaling axis promotes the invasion of A549, 95D and H292 lung cancer cells *in vitro* (22), implicating the CXCL16-CXCR6 signaling axis in the development of lung cancer. However, whether there is variability in CXCL16 and CXCR6 expression between patients with lung cancer with different clinicopathological features has not yet been investigated, to the best of our knowledge. In the present clinical retrospective study, the association of t-CXCL16, t-CXCR6 and s-CXCL16 levels with clinicopathological features was investigated in patients with NSCLC. The data from the present study provide new insights into potential biomarkers for the early detection of lung cancer, and into targeted therapy and postoperative follow-up for patients with NSCLC.

Materials and methods

Tissue sample collection. All procedures involving participants in the present study were approved by the Human Research Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China), and written informed consent was provided by all participants. Tissue collection was performed as previously described (22). Briefly, human lung cancer tissue (58 cases) and the adjacent normal lung tissue (20 cases) was obtained from patients who underwent pulmonary lobe resection or pneumonectomy at Zhongnan Hospital of Wuhan University between August 2013 and September 2014. Two experienced pathologists performed the identification of the pathological type and differentiation degree of NSCLC. Tumor (T) stage was determined according to the seventh edition of the tumor-node-metastasis (TNM) staging system of the International Association for the Study of Lung Cancer (IASLC) in 2009 (23). The recruitment criteria for patients included a pathological diagnosis of primary NSCLC, without any other primary tumor history, intact medical records and follow-up data. The exclusion criteria were preoperative chemotherapy, radiotherapy, biological therapy or immunotherapy. The clinicopathological characteristics of the patients included in the present study are provided in Table I.

Immunohistochemistry (IHC). IHC was performed as previously described (17,22). rabbit polyclonal CXCL16 (cat. no. ab101404; dilution, 1:100) and rabbit polyclonal CXCR6 (cat. no. ab8023; dilution, 1:100) antibodies from Abcam (Cambridge, MA, USA) were used as the primary antibodies in the study. They were validated by the manufacturer for immunohistochemistry on paraffin-embedded material. The tissues were fixed with formalin and embedded in paraffin. The 4- μ m tissue sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the sections in 0.01 mol/l citrate buffer, pH 6.0, before microwave

heating for 15 min at 400 W. Following antigen retrieval, 0.3% H₂O₂ for 15 min in PBS was used to block endogenous peroxidase activity in the 4- μ m tissue sections. Following treatment with citrate buffer (MaiXin Biotechnology, Co., Ltd., Fuzhou, China) to clear non-specific binding, the sections were incubated overnight at 4°C with 25 μ g/ml CXCR6 or 20 μ g/ml CXCL16 primary antibodies. The CXCR6 and t-CXCL16 molecules were visualized by adding horseradish peroxidase (HRP)-labeled mouse anti-rabbit IgG (cat. no. KIT-9901, dilution, 1:100, MaiXin Biotechnology, Co., Ltd., Fuzhou, China), which were included in a detection reagent kit (Elivision™ plus Polyer HRP (Mouse/Rabbit) IHC Kit, cat. no. KIT-9901, MaiXin Biotechnology, Co., Ltd., Fuzhou, China) at 37°C for 15 min. Then 3,3-diaminobenzidine tetrahydrochloride was used for signal detection and Harris hematoxylin was used as a counterstain. The reagents for immunohistochemical analysis, including the citrate buffer, H₂O₂, detection kit, DAB and hematoxylin were purchased from MaiXin Biotechnology, Co. Ltd. A total of 10 μ g/ml rabbit isotype immunoglobulin G (cat. no. AG-0021; dilution, 1:50; Dingguo Bio Co., Ltd., Shanghai, China) was used as a negative control.

Scoring of immunohistochemistry (IHC). The IHC scoring was performed blindly using a telepathology system without knowledge of the associated clinical information, including tumor grade, tumor size and clinical outcome (17,22). The tissue sections were assigned scores respectively based on the intensity of immunostaining and the percentage of positively stained cells. The immunostaining intensity was observed and scored as follows: No staining (score, 0), light yellow staining (score, 1), light brown staining (score, 2) or brown staining (score, 3). The percentage of positively stained cells was scored as follows: 5% (score, 0), 5-25% (score, 1), 26-50% (score, 2), 51-75% (score, 3) or >75% (score, 4). The sum of the immunostaining intensity score and the score for the percentage of positive cells was the overall score of every tissue slice, which was defined as follows: <2, negative expression(-); \geq 2 positive expression; 2-3, weak expression(+); 4-5, moderate expression(++); and 6-7 as strong expression(+++) (17,22).

ELISA. A total of 2 ml venous blood was collected using 10 ml syringe in morning, then spaced into vacuum packing tubes without anticoagulant. The samples were allowed to stand for 30 min. Subsequent to low-speed centrifugal 1,200 x g for 10 min, the supernatant was collected into EP tubes, and then centrifuged at 19,200 x g for 10 min. Now the supernatant is the serum samples. The blood sera from 58 patients with NSCLC and 32 normal volunteers (17 were men and 13 were female, age range 46-72 years) were collected from between August 2013 and September 2014 and stored at -80°C until the ELISA analysis was performed. The amount of s-CXCL16 in each sample was measured using a human CXCL16 ELISA kit (cat. no. F00514; Shanghai Westang Bio-Tech Co., Ltd., Shanghai, China), according to the manufacturer's protocol. The CXCL16 assay kit demonstrated a sensitivity of 40 pg/ml and an intra-assay coefficient of variation of <12% (22).

Statistical analysis. The data were analyzed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). The results of the ELISA are presented as the mean \pm standard error and

Table I. Clinicopathological characteristics of the patients with non-small cell lung cancer included in the present study.

Clinicopathological characteristic	No. of patients	Proportion of patients (%)
Age (years)		
<60	32	55.2
≥60	26	44.8
Gender		
Male	42	72.4
Female	16	27.6
Pathological type		
AC	32	55.2
SC	22	37.9
ASC	4	6.9
Differentiation degree		
Low	15	25.9
Moderate	30	51.7
High	13	22.4
TMN stage		
I-II	30	51.7
III-IV	28	48.3
Smoking		
+	37	63.8
-	21	36.2
Pleural invasion		
+	4	6.9
-	54	93.1
Lymph node metastasis		
+	34	58.6
-	24	41.4

AC; adenocarcinoma, SC; squamous carcinoma, ASC; adenosquamous carcinoma.

were assessed using a Student's t-test. The association between CXCL16-CXCR6 expression and clinicopathological features was analyzed with a χ^2 test, Fisher's exact test and Spearman rank correlation coefficient analysis. A univariate analysis was performed using the Kaplan-Meier estimator method and a log-rank test. The median survival time was calculated using SPSS v17.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics of patients with NSCLC. The age of patients with NSCLC ranged from 43-80 years and the average age was 58.95 ± 9.84 years (Table I). A total of 72.4% of patients with NSCLC were male and 27.6% were female. The pathological types were as follows: Adenocarcinoma (AC; 32 cases), squamous carcinoma (SC; 22 cases) and adenosquamous carcinoma (ASC; 4 cases). Differentiation degrees included low (15 cases), medium (30 cases) and high (13 cases).

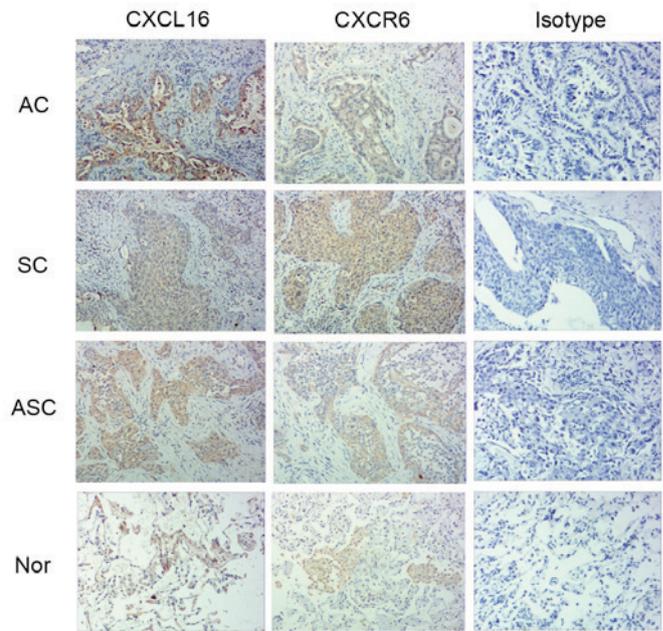


Figure 1. Expression of CXCL16 and CXCR6 in human primary non-small cell lung cancer tissue. Immunohistochemical staining revealed CXCL16 and CXCR6 expression in the cytoplasm and membrane of human primary non-small cell lung cancer cells. There was no evidence for nonspecific staining with the control antibody (isotype). The images were representative of the experiments. Magnification, x200. AC, adenocarcinoma; SC, squamous carcinoma; ASC, adenosquamous carcinoma; Nor, normal lung tissue; CXCR6, C-X-C chemokine receptor type 6; CXCL16, C-X-C motif chemokine 16.

A total of 30 patients were identified to have stage I-II NSCLC and 28 patients were identified to have stage III-IV NSCLC. Among the 58 patients there were 37 smokers, 4 patients with pleural invasion and 34 patients with lymph node metastasis.

Association between t-CXCL16 and t-CXCR6 expression in patients with NSCLC. IHC was performed to detect the expression of t-CXCL16 and t-CXCR6 protein in human lung tissues derived from primary NSCLCs (Table II). t-CXCR6- and t-CXCL16-specific staining was clearly observed in the cytoplasm and membrane of the primary lung cancer cells (Fig. 1). In normal lung tissue, t-CXCL16 and t-CXCR6 were primarily restricted to the alveolar epithelial cells and inflammatory cells. According to the scoring of IHC, the positive expression rate was defined as that the ratio between the positive expression case (overall score of the tissue slice ≥ 2) and all cases in the same group. No significant difference was identified between the positive expression rate of t-CXCL16 (72.41% of cases) and that of t-CXCR6 protein (65.52% of cases) ($P = 0.442$; Table II). Among the 58 patients with NSCLC, there were 38 cases (65.52%) that co-expressed t-CXCL16 and t-CXCR6 (data not shown).

Association between t-CXCL16/t-CXCR6 expression and the clinicopathological characteristics of patients with NSCLC (Table III). No significant difference was observed between the positive expression rate of t-CXCL16 in the <60 years subgroup compared with that of the ≥ 60 years subgroup (78.13 vs. 65.38%; $P = 0.280$). The same was true for t-CXCR6 in the <60 compared with the ≥ 60 years old groups (71.88 vs. 57.69%;

Table II. Positive expression rate of CXCL16 and CXCR6 in non-small cell human lung cancer tissues.

Protein	No. of patients	Expression		Positive expression rate (%)	P-value
		Positive	Negative		
CXCL16	58	42	16	72.41	0.422
CXCR6	58	38	20	65.52	

CXCL16, C-X-C motif chemokine 16; CXCR6, C-X-C chemokine receptor type 6. According to the scoring of IHC, the positive expression rate was defined as that the ratio between the positive expression case (overall score of the tissue slice ≥ 2) and all cases in the same group.

Table III. Association between CXCL16/CXCR6 expression and the clinicopathological characteristics of patients with non-small cell lung cancer.

Clinicopathological characteristic	Subgroup	Total cases	CXCL16				CXCR6			
			+	-	PR (%)	P-value	+	-	PR (%)	P-value
Age (years)	<60	32	25	7	78.13	0.280	23	9	71.88	0.258
	≥ 60	26	17	9	65.38		15	11	57.69	
Gender	Male	42	30	12	71.43	1.000	27	15	64.29	0.749
	Female	16	12	4	75.0		11	5	68.75	
Pathological type	AC	32	24	8	75.0	0.895	21	11	65.63	1.000
	SC	22	15	7	68.18		14	8	63.64	
	ASC	4	3	1	75.0		3	1	75.0	
Differentiation degree	Low	15	12	3	80.0	0.799	10	5	66.67	0.927
	Moderate	30	21	9	70.0		19	11	63.33	
	High	13	9	4	69.23		9	4	69.23	
TMN stage	I-II	30	18	12	60.0	0.029 ^a	16	14	53.33	0.043 ^a
	III-IV	28	24	4	85.71		22	6	78.57	
Smoking	+	37	37	0	100.0	<0.001 ^a	24	13	64.86	0.89
	-	21	5	16	23.81		14	7	66.67	
Pleural invasion	+	4	4	0	100.0	0.480	3	1	75.0	1.000
	-	54	38	16	70.37		35	19	64.81	
Lymph node metastasis	+	34	30	4	91.18	0.001 ^a	27	7	79.41	0.008 ^a
	-	24	12	12	50.0		11	13	45.83	

^aP<0.05. AC, adenocarcinoma; SC, squamous carcinoma; ASC, adenosquamous carcinoma; PR, positive expression rate. -, negative, +, positive. PR%: the ratio between the positive expression patient (overall score of the tissue slice ≥ 2) and all patients in the same group.

P=0.258). No significant differences were identified between the positive expression rates of t-CXCL16 (71.43 vs. 75%; P=0.28) or t-CXCR6 (64.29 vs. 68.75%; P=0.749) between the male subgroup and the female subgroups, respectively.

Additionally, no significant differences were identified between the positive expression rates of t-CXCL16 or t-CXCR6 in different pathological types of NSCLC (Table III). For t-CXCL16, the positive expression rates were 75, 68.18 and 75% in the AC, SC and ASC subgroups (P=0.895), respectively. For t-CXCR6, the positive expression rates were 65.63, 63.64 and 75% in the AC, SC and ASC subgroups (P=1.000), respectively. Similar results were observed when comparing subgroups of patients with NSCLC that was differentiated to different degrees. For t-CXCL16, the positive expression rates were 80, 70 and 69.23% in the low, medium and high

subgroups (P=0.799), respectively. For t-CXCR6, the positive expression rates were 66.67, 63.33 and 69.23% in the low, medium and high subgroups (P=0.927), respectively. No significant differences were identified between the expression rates of t-CXCL16 (100 vs. 70.37%, P=0.480) or t-CXCR6 (75 vs. 64.81%, P=1.000) in the pleural invasion and non-pleural invasion subgroups.

When patients were grouped according to TNM stage, the positive expression rate of t-CXCL16 (60 vs. 85.71%, P=0.029) and t-CXCR6 (53.33 vs. 78.57%, P=0.043) in stage I-II was significantly lower compared with that of stage III-IV (Table III). Furthermore, the expression rate of t-CXCL16 (91.18 vs. 50%, P=0.001) and t-CXCR6 (79.41 vs. 45.83%, P=0.008) in the lymph node metastasis subgroup was significantly higher compared with that of the corresponding

Table IV. Concentration of s-CXCL16 in patients with NSCLC and normal volunteers.

Group	No. of patients	s-CXCL16 (pg/ml)	P-value
NSCLC	58	572.82±116.05	<0.001
Normal volunteers	32	329.47±135.38	

NSCLC, non-small cell lung cancer; s-CXCL16, serum C-X-C motif chemokine 16.

Table V. Expression pattern of s-CXCL16 and t-CXCL16 in patients with non-small cell lung cancer.

		t-CXCL16 concentration				Total
		-	+	++	+++	
s-CXCL16 concentration	Low	11	10	10	3	34
	High	5	12	5	2	24
Total		16	22	15	5	

s-CXCL16, serum C-X-C motif chemokine 16; t-CXCL16, tissue C-X-C motif chemokine 16. -, negative expression; +, weak expression; ++, moderate expression; +++, strong expression. s-CXCL16 concentration of 58 patients in NSCLC was divided into two subgroups: High (≥ 572.82 pg/ml, 24 patients) and low (< 572.82 pg/ml, 34 patients).

non-lymph node metastasis subgroup. Although no significant difference in t-CXCR6 expression was identified between the smoking and non-smoking subgroups (64.86 vs. 66.67%, $P=0.890$), the positive expression rate of t-CXCL16 in the smoking subgroup was significantly higher compared with that of the non-smoking subgroup (100 vs. 23.81%, $P<0.001$).

Association between s-CXCL16 concentration and the clinicopathological characteristics of patients with NSCLC. An ELISA was performed to compare the concentration of s-CXCL16 in patients with NSCLC and normal volunteers. As illustrated in Table IV, the s-CXCL16 concentration in patients with NSCLC was significantly higher compared with that of the normal volunteers (572.82±116.05 vs. 329.47±135.38 pg/ml; $P<0.001$). According to the average s-CXCL16 concentration, patients with NSCLC were further divided into two subgroups as follows: High (≥ 572.82 pg/ml, 24 cases) and low (< 572.82 pg/ml, 34 patients; Tables V and VI). Among the s-CXCL16 high concentration group containing 24 cases in total, there were 5 patients that did not express t-CXCL16, 12 patients with weak expression, 5 with moderate expression and strong expression in 2 patients (Table V). Compared with t-CXCR6, the high group included 8 patients with negative expression, 11 patients with weak expression, only one patient with moderate expression and 4 with strong expression

Table VI. Expression pattern of s-CXCL16 and t-CXCR6 in patients with non-small cell lung cancer.

		t-CXCR6 concentration				Total
		-	+	++	+++	
s-CXCL16 concentration	Low	12	11	3	8	34
	High	8	11	1	4	24
Total		20	22	4	12	

s-CXCL16, serum C-X-C motif chemokine 16; t-CXCR6, tissue C-X-C chemokine receptor type 6. -, negative expression; +, weak expression; ++, moderate expression; +++, strong expression. s-CXCL16 concentration of 58 patients in NSCLC was divided into two subgroups: High (≥ 572.82 pg/ml, 24 cases) and low (< 572.82 pg/ml, 34 cases).

(Table VI). Prominent significant differences were identified between the t-CXCL16 (72.41 vs. 41.38%; Chi-square=11.389; $P=0.001$) or t-CXCR6 (65.52 vs. 41.38%; Chi-square=6.791; $P=0.009$) IHC positive expression rates and the s-CXCL16 high-level subgroup (Not shown in Tables V and VI).

The associations between s-CXCL16 levels (high or low) and clinicopathological characteristics of the patients are illustrated in Table VII. There was no correlation between age and the percentage of cases with a high level of s-CXCL16 [< 60 years) vs. 46.15% (≥ 60 years); $P=0.506$]. No significant difference was identified between the percentage of male patients with a high s-CXCL16 level and the percentage of female patients with a high s-CXCL16 level (40.48 vs. 43.75%; $P=0.821$).

When grouped according to pathological type, the percentage of patients with a high s-CXCL16 level was 34.38, 50 and 50% in the AC, SC and ASC subgroups, respectively ($P=0.487$; Table VII). No significant difference was identified between the percentages of patients with a high s-CXCL16 level in different differentiation degree subgroups [60% (low) vs. 33.33% (medium) vs. 38.46% (high); $P=0.224$]. Additionally, the presence or absence of smoking (37.84 vs. 47.62%; $P=0.467$), pleural invasion (50 vs. 40.74%; $P=1.000$) or lymph node metastasis (44.12 vs. 37.5%; $P=0.641$) had no effect on the expression level of s-CXCL16 in patients with NSCLC. However, when grouped according to TNM stage, the percentage of patients with a high s-CXCL16 level in the stage I-II subgroup (26.67%) was significantly lower compared with that of the stage III-IV (57.14%) subgroup ($P=0.019$).

Effects of t-CXCL16, t-CXCR6 and s-CXCL16 expression on patients' prognosis. Professional personnel performed a follow-up every 4 months between January 2014 and January 2015. The follow-up rate and mortality rate were 100% (58/58) and 13.79% (8/58), respectively. Within the time period of the present study, the survival time were 4-18 months and the mean survival time was 16.6 months (data not shown). No significant difference was identified between the survival rate of the t-CXCL16-positive group (88.1%) compared with the t-CXCL16-negative group (81.25%) (log-rank, 0.008;

Table VII. Association between s-CXCL16 concentration and the clinicopathological characteristics of patients with non-small cell lung cancer.

Group	Subgroup	Total no. of cases	s-CXCL16			P-value
			High	Low	PR ((%)	
Age (years)	<60	32	12	20	37.50	0.506
	≥60	26	12	14	46.15	
Gender	Male	42	17	25	40.48	0.821
	Female	16	7	9	43.75	
Pathological type	AC	32	11	21	34.38	0.487
	SC	22	11	11	50.00	
	ASC	4	2	2	50.00	
Differentiation degree	Low	15	9	6	60.00	0.224
	Moderate	30	10	20	33.33	
	High	13	5	8	38.46	
TMN stage	I-II	30	8	22	26.67	0.019 ^a
	III-IV	28	16	12	57.14	
Smoking	+	37	14	23	37.84	0.467
	-	21	10	11	47.62	
Pleural invasion	+	4	2	2	50.00	1.000
	-	54	22	32	40.74	
Lymph node metastasis	+	34	15	19	44.12	0.614
	-	24	9	15	37.50	

^aP<0.05. AC, adenocarcinoma; SC, squamous carcinoma; ASC, adenosquamous carcinoma; PR: positive rate; s-CXCL16, serum C-X-C motif chemokine 16. PR%=the ratio between High s-CXCL16 concentration cases and All cases.

P=0.931; Fig. 2A). Additionally, no significant difference was identified between the t-CXCR6-positive (84.21%) and the t-CXCR6-negative (90%) groups (log-rank, 1.559; P=0.212; Fig. 2B). The survival rate of patients with NSCLC [87.5% (high subgroup) vs. 85.29% (low subgroup)] was not associated with the s-CXCL16 level (log-rank, 0.068; P=0.795; Fig. 2C). The median survival time was not obtained as the follow-up time was relatively short and all the survival rates were >50%. Therefore, it was difficult to obtain the median survival time with the method used in the present study.

Discussion

The aim of the present study was to investigate the role of the CXCL16-CXCR6 signaling axis in the progression and metastasis of human lung cancer. The expression of t-CXCL16, t-CXCR6 and s-CXCL16 was measured in 58 patients with NSCLC, and the association between these expression levels and different clinicopathological features was explored. In accordance with a previous study (22), t-CXCR6- and t-CXCL16-specific staining was clearly observed in the cytoplasm and membrane of the primary lung cancer cells (Fig. 1). The data revealed that t-CXCL16 and t-CXCR6 were co-expressed in human primary NSCLC tissue, and no significant difference was identified between the positive expression rates of t-CXCL16 and t-CXCR6 (P=0.442). This

expression pattern of t-CXCL16 and t-CXCR6 is similar to that observed in a previous study (22). A total of 91 samples were investigated across the present study and this previous study. Thus, the co-expression of CXCL16 and CXCR6 may serve an important role in the development of human lung cancer.

Age and gender, and the pathological type and differentiation degree of NSCLC, had no significant effect on t-CXCL16 or t-CXCR6 expression in NSCLC tissue. However, there was a significant difference between the positive expression rates of t-CXCL16 (P=0.029) and t-CXCR6 (P=0.043) of the stage III-IV and I-II TNM subgroups. The same result was observed when the patients were grouped according to the occurrence of lymphatic metastasis. The positive expression rates of t-CXCL16 (P=0.001) and t-CXCR6 (P=0.008) of the lymph node metastasis subgroup were significantly higher compared with that of the corresponding non-lymph node metastasis subgroup. Previous studies have demonstrated that the CXCL16-CXCR6 signaling axis promotes the viability and invasiveness of lung cancer cell lines *in vitro* (22). Recent *in vivo* experiments from our group have demonstrated that blocking the CXCL16-CXCR6 signaling axis effectively inhibits tumor formation in nude mice (Hu *et al*, unpublished data). This previous data, and the data from the present study, suggest that the CXCL16-CXCR6 signaling axis is associated with human lung tumor metastasis.

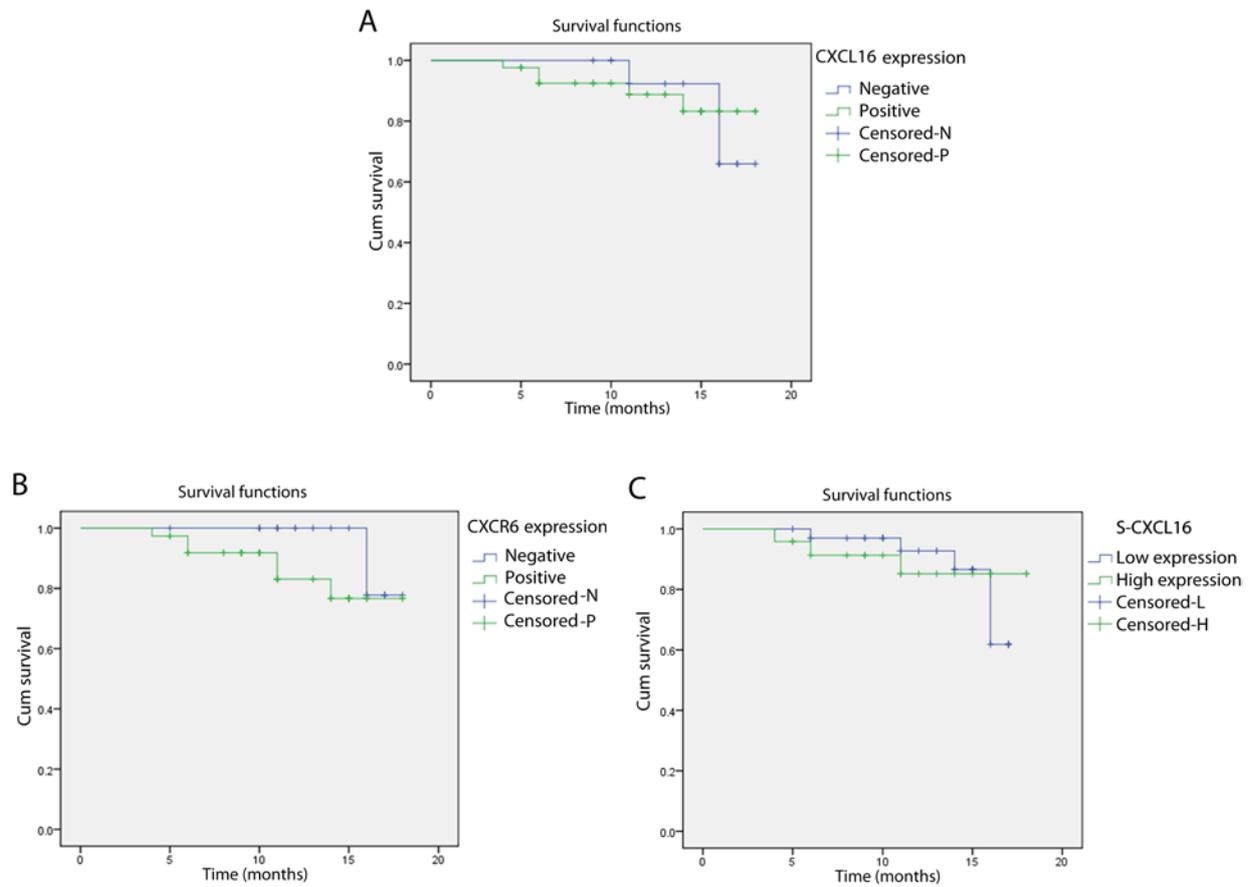


Figure 2. Survival curves of patients with NSCLC according to t-CXCL16, t-CXCR6 and s-CXCL16 expression. The specific survival time of patients with NSCLC according to their expression of (A) t-CXCL16 in human primary lung cancer tissues (Kaplan-Meier estimator; log-rank, 0.008; $P=0.931$), (B) t-CXCR6 in human primary lung cancer tissues (Kaplan-Meier estimator; log-rank, 1.559; $P=0.212$) and (C) soluble CXCL16 in blood serum (Kaplan-Meier estimator; log-rank, 0.068; $P=0.795$). NSCLC, non-small cell lung cancer; CXCR6, C-X-C chemokine receptor type 6; CXCL16, C-X-C motif chemokine 16; t, transmembrane.

The expression pattern of t-CXCL16 was different in the smoking subgroup when compared with the non-smoking subgroup, and also when comparing the TNM stage and lymphatic metastasis status. There was no significant difference in t-CXCR6 expression between the smoking subgroup and the non-smoking subgroup ($P=0.89$). However, all patients with NSCLC from the smoking subgroup expressed t-CXCL16 at a significantly higher level compared with those in the non-smoking subgroup ($P<0.001$). Under normal conditions, CXCL16 is constitutively expressed by human bronchial epithelial cells, which is important for the homeostatic regulation of T cells and resistance to external pathogens (24). During an inflammatory response, including the response to regular smoking, CXCL16 can be upregulated (16,25). The role of inflammation in the tumor microenvironment during tumorigenesis has been investigated (26,27). It has been demonstrated in prostate cancer that inflammatory cytokines derived from adjacent infiltrating CXCR6-positive T cells can stimulate the production of CXCL16 by cancer cells, and that CXCL16 then further enhances the growth and proliferation of CXCR6-expressing cancer cells and primary T cells (16). Thus, the smoking-associated inflammatory microenvironment, together with an abnormal increase of CXCL16, may contribute to the high risk of lung cancer for smokers.

CXCL16 can exist in a t and s form. Thus, in the present study, the concentration of s-CXCL16 was examined in patients with NSCLC and in normal volunteers. The s-CXCL16 level in patients with NSCLC was significantly increased compared with that in the normal volunteers ($P<0.001$). No significant differences were identified between the expression levels of s-CXCL16 among the age, gender, pathological type, differentiation degree, smoking, pleural invasion or lymph node metastasis subgroups. This may be due to the small sample size of the present study. However, the level of s-CXCL16 in the stage III-IV TNM subgroup was significantly higher compared with that of the corresponding stage I-II TNM subgroup ($P=0.019$). The results from the present study suggest that s-CXCL16 levels are a novel biomarker for the early detection and grading of NSCLC. However, further studies investigating the role that s-CXCL16 serves in tumor metastasis are required, as the level of s-CXCL16 may not be representative of the level of t-CXCL16 in the lung.

One limitation of the present study is the small cohort size, which did not allow for a thorough survival analysis. Besides, the follow-up period was short and most patients were still alive at the end of this study, so we cannot get the median survival time of the patients, and further study should be conducted for the survival effect. Although a previous study has investigated the association between CXCL16 expression and the survival

of patients with lung cancer (28), further studies investigating the prognostic impact of CXCL16 and CXCR6 expression in larger multicenter cohorts of patients with NSCLC are required. Additionally, pleural invasion typically occurs in the advanced stages of NSCLC when surgery is not appropriate. Thus, in the present study's patient cohort, there were only 4 patients with pleural metastasis. The underlying molecular mechanisms of pleural metastasis in NSCLC require further investigation.

In conclusion, the present study demonstrated that t-CXCL16 and t-CXCR6 are co-expressed in human NSCLC. The TNM stage and lymph node metastasis status were positively correlated with the expression levels of t-CXCL16 and t-CXCR6, suggesting that the CXCL16-CXCR6 signaling axis serves a role in the development and metastasis of lung cancer. Additionally, there was a significant increase in s-CXCL16 levels in patients with NSCLC, suggesting that s-CXCL16 could be used as a biomarker for the early detection of lung cancer. In addition, the expression of t-CXCL16 was significantly increased in patients with NSCLC that smoked compared with patients that did not smoke, which provides insight into a potential underlying molecular mechanism for the high risk of lung cancer in smokers.

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References

- Bray F, Ren JS, Masuyer E and Ferlay J: Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *Int J Cancer* 132: 1133-1145, 2013.
- Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics, 2014. *CA Cancer J Clin* 64: 9-29, 2014.
- Wood SL, Pernemalm M, Crosbie PA and Whetton AD: The role of the tumor-microenvironment in lung cancer-metastasis and its relationship to potential therapeutic targets. *Cancer Treat Rev* 40: 558-566, 2014.
- Gerber PA, Hippe A, Buhren BA, Müller A and Homey B: Chemokines in tumour associated angiogenesis. *Biol Chem* 390: 1213-1223, 2009.
- Raman D, Baugher PJ, Thu YM and Richmond A: Role of chemokines in tumor growth. *Cancer Lett* 256: 137-165, 2007.
- Vandercappellen J, Van Damme J and Struyf S: The role of CXC chemokines and their receptors in cancer. *Cancer Lett* 267: 226-244, 2008.
- Sarvaiya PJ, Guo D, Ulasov I, Gabikian P and Lesniak MS: Chemokines in tumor progression and metastasis. *Oncotarget* 4: 2171-2185, 2013.
- Keeley EC, Mehrad B and Strieter RM: CXC chemokines in cancer angiogenesis and metastases. *Adv Cancer Res* 106: 91-111, 2010.
- Matloubian M, David A, Engel S, Ryan JE and Cyster JG: A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat Immunol* 1: 298-304, 2000.
- Shimaoka T, Kume N, Minami M, Hayashida K, Kataoka H, Kita T and Yonehara S: Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages. *J Biol Chem* 275: 40663-40666, 2000.
- Wilbanks A, Zondlo SC, Murphy K, Mak S, Soler D, Langdon P, Andrew DP, Wu L and Briskin M: Expression cloning of the STRL33/BONZO/TYMSTR ligand reveals elements of CC, CXC and CX3C chemokines. *J Immunol* 166: 5145-5154, 2001.
- Hattermann K, Ludwig A, Gieselmann V, Held-Feindt J and Mentlein R: The chemokine CXCL16 induces migration and invasion of glial precursor cells via its receptor CXCR6. *Mol Cell Neurosci* 39: 133-141, 2008.
- Hara T, Katakai T, Lee JH, Nambu Y, Nakajima-Nagata N, Gonda H, Sugai M and Shimizu A: A transmembrane chemokine, CXC chemokine ligand 16, expressed by lymph node fibroblastic reticular cells has the potential to regulate T cell migration and adhesion. *Int Immunol* 18: 301-311, 2006.
- Huang Y, Zhu XY, Du MR, Wu X, Wang MY and Li DJ: Chemokine CXCL16, a scavenger receptor, induces proliferation and invasion of first-trimester human trophoblast cells in an autocrine manner. *Hum Reprod* 21: 1083-1091, 2006.
- Deng L, Chen N, Li Y, Zheng H and Lei Q: CXCR6/CXCL16 functions as a regulator in metastasis and progression of cancer. *Biochim Biophys Acta* 1806: 42-49, 2010.
- Darash-Yahana M, Gillespie JW, Hewitt SM, Chen YY, Maeda S, Stein I, Singh SP, Bedolla RB, Peled A, Troyer DA, *et al*: The chemokine CXCL16 and its receptor, CXCR6, as markers and promoters of inflammation-associated cancers. *PLoS One* 4: e6695, 2009.
- Hu W, Zhen X, Xiong B, Wang B, Zhang W and Zhou W: CXCR6 is expressed in human prostate cancer in vivo and is involved in the in vitro invasion of PC3 and LNCap cells. *Cancer Sci* 99: 1362-1369, 2008.
- Meijer J, Ogink J, Kreike B, Nuyten D, de Visser KE and Roos E: The chemokine receptor CXCR6 and its ligand CXCL16 are expressed in carcinomas and inhibit proliferation. *Cancer Res* 68: 4701-4708, 2008.
- Gutwein P, Schramme A, Sinke N, Abdel-Bakky MS, Voss B, Obermüller N, Doberstein K, Koziolok M, Fritzsche F, Johannsen M, *et al*: Tumoural CXCL16 expression is a novel prognostic marker of longer survival times in renal cell cancer patients. *Eur J Cancer* 45: 478-489, 2009.
- Ou DL, Chen CL, Lin SB, Hsu CH and Lin LI: Chemokine receptor expression profiles in nasopharyngeal carcinoma and their association with metastasis and radiotherapy. *J Pathol* 210: 363-373, 2006.
- Lee JT, Lee SD, Lee JZ, Chung MK and Ha HK: Expression analysis and clinical significance of CXCL16/CXCR6 in patients with bladder cancer. *Oncol Lett* 5: 229-235, 2012.
- Hu W, Liu Y, Zhou W, Si L and Ren L: CXCL16 and CXCR6 are coexpressed in human lung cancer in vivo and mediate the invasion of lung cancer cell lines in vitro. *PLoS One* 9: e99056, 2014.
- Groome PA, Bolejack V, Crowley JJ, Kennedy C, Krasnik M, Sobin LH and Goldstraw P: IASLC International Staging Committee; Cancer Research and Biostatistics; Observers to the Committee; Participating Institutions: The IASLC lung cancer staging project: Validation of the proposals for revision of the T, N and M descriptors and consequent stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumours. *J Thorac Oncol* 2: 694-705, 2007.
- Day C, Patel R, Guillen C and Wardlaw AJ: The chemokine CXCL16 is highly and constitutively expressed by human bronchial epithelial cells. *Exp Lung Res* 35: 272-283, 2009.
- Sallusto F, Mackay CR and Lanzavecchia A: The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol* 18: 593-620, 2000.
- Kamp DW, Shacter E and Weitzman SA: Chronic inflammation and cancer: The role of the mitochondria. *Oncology (Williston Park)* 25: 400-410, 413, 2011.
- Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Goltsovich-Pyest E, Urieli-Shoval S, Galun E and Ben-Neriah Y: NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 431: 461-466, 2004.
- Hald SM, Kiselev Y, Al-Saad S, Richardsen E, Johannessen C, Eilertsen M, Kilvaer TK, Al-Shibli K, Andersen S, Busund LT, *et al*: Prognostic impact of CXCL16 and CXCR6 in non-small cell lung cancer: Combined high CXCL16 expression in tumor stroma and cancer cells yields improved survival. *BMC Cancer* 15: 441, 2015.