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## Expression of Chemokine XCL2 and CX3CL1 in Lung Cancer

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Data Interpretation D  
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**Background:** Chemokines are a family of small proteins secreted by cells with chemotactic activity, and they play important roles in cell adhesion. However, the expression of chemokine XCL2 and CX3CL1 in lung cancers in different pathological stages remains unclear.

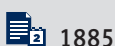
**Material/Methods:** XCL2 and CX3CL1 expression in lung cancers and adjacent non-cancerous tissues was detected by quantitative PCR and ELISA. The relative expression of both chemokines in lung cancers in different pathological stages was compared by immunohistochemical assay.

**Results:** The relative expression level of XCL2 and CX3CL1 in lung cancer was significantly higher compared with adjacent normal tissues ( $P < 0.001$ ). The expression level of both chemokines was significantly increased with higher pathological stages, as indicated by immunohistochemical assay ( $P < 0.05$  or  $P < 0.001$ ). Their expression level in cancers with higher numbers of metastatic lymph nodes was also significantly increased compared with cancers with lower numbers of metastatic lymph nodes ( $P < 0.05$  or  $P < 0.001$ ).

**Conclusions:** The expression of XCL2 and CX3CL1 increases with increasing degree of malignancy, indicating that both chemokines might be important targets in gene therapy for lung cancer.

**MeSH Keywords:** **Chemokine CX3CL1 • Chemokine CXCL2 • Lung Neoplasms**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/895985>



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## Background

Lung cancer is the most common fatal malignancy worldwide. There are 2 major pathological types of lung cancer, which account for approximately 85% of lung cancers: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [1]. Lung cancer can be further divided into 3 histological types: squamous cell carcinoma, adenocarcinoma, and large cell lung cancer. Currently, the primary treatment method for lung cancer is surgery, supplemented by comprehensive treatment with chemotherapy, radiation therapy, and targeted therapy. Nevertheless, the 5-year survival rate for lung cancer is only 15% [2]. Lung cancer does not usually cause any symptoms in the early stages of the disease, and thus is often diagnosed in late stages. Therefore, the identification of early diagnostic markers is important for the diagnosis and treatment of lung cancer.

Chemokines are a family of small proteins of molecular weights ranging from 8 to 17 kDa, with chemotactic activity. Studies have shown that chemokines guide cell migration along a concentration gradient during inflammatory reactions. The chemokine superfamily can be divided into the following branches: C, CC, CXC, and CXXXC. Chemokines play important roles in the occurrence and development of cancers due to their chemotactic activity in cancer cells. However, there have been few studies of chemokines in lung cancer [3]. XCL2 is a small protein of the XC chemokine family, and is closely associated with another chemokine, XCL1. It is mainly expressed in activated T cells, and is also expressed at low levels in inactivated T cells. The CX3CL1 gene is located on human chromosome 16 [4,5]. In contrast to the typical structure of other chemokines, CX3CL1 has different spacing of the characteristic N-terminal cysteines. There are 3 amino acids separating the initial pair of cysteines in CX3CL1, with none in CC chemokines and only 1 intervening amino acid in CXC chemokines. Soluble CX3CL1 has the ability to recruit T cells and monocytes, and thus enhances binding and adhesion capacity of cells. CXCL1 can affect cell adhesion and migration through its receptor, CX3CR1 [6,7]. Studies have shown that CX3CL1 is highly expressed in the hippocampus region during short-term memory, which may be regulated by glutamatergic neurotransmission [8,9]. The interaction between CX3CL1 and glutamate led us to investigate the expression of XCL2 and CX3CL1 in lung cancer.

## Material and Methods

### Sample collection

A total of 38 tumor samples were collected from 18 male and 20 female patients with NSCLC who underwent surgical resection

in Zhejiang Provincial People's Hospital between July 2014 and December 2014. A total of 20 adjacent non-cancerous tissues were used as controls. Based on the TNM classification for lung cancer staging (2009) by the Union for International Cancer Control (UICC), the clinical staging of selected patients was as follows: 23 cases of squamous cell carcinoma, and 15 cases of adenocarcinoma; TEM staging: 21 cases of stage I-II, and 17 cases of stage III-IV; lymph node metastasis for lung cancer staging: 24 cases of N0, 7 cases of N1, and 7 cases of N2.

The study protocol was approved by the Research Ethics Committee of Zhejiang Provincial People's Hospital, and all patients gave their informed consent before study commencement.

### Reagents

XCL2 and CX3CL1 antibodies were purchased from Cell Signaling Technology ((Beverly, MA, USA). RPMI-1640 medium and fetal bovine serum was purchased from Hyclone, Inc. (Logan, UT, USA).

### Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol RNA extraction kit (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription kits were purchased from TaKaRa. Aliquots of tissue or serum were mixed with equal amounts of TRIzol, and centrifuged at 12 000 rpm at 4°C for 10 min. The supernatant was transferred into a new Eppendorf (EP) tube, chilled on ice for 5 min, and mixed with 200 µL of chloroform. The mixture was vortexed for 15 s and centrifuged at 12 000 rpm at 4°C for 15 min. The top layer was transferred into a new EP tube and mixed with an equal volume of isopropanol. The mixture was allowed to precipitate at room temperature for 10 min, and centrifuged at 12 000 rpm/min at 4°C for 10 min. The supernatant was discarded. The remnant was mixed with 75% ethanol and centrifuged at 7500 rpm at 4°C for 5 min. The supernatant was carefully removed. RNA was air-dried and dissolved in 20 µL of DEPC-treated water. The expression of XCL2 and CX3CL1 was detected using nucleic acid dye. Small RNAs were isolated by use of a small RNA purification kit. RNAs were then transcribed into cDNA, which was further amplified using a one-step RT-PCR kit. Primers were added into 10 µL of PCR reaction system. The reaction conditions were 94°C for 45 s, followed by 35 cycles of refolding at 59°C for 45 s and extension at 72°C. The primers are listed in Table 1.

### Immunohistochemical staining of XCL2 and CX3CL1 in lung cancer

Sections were dewaxed using the conventional dewaxing procedure. Briefly, sections were treated sequentially with xylene, absolute ethanol, and 95%, 90%, 85%, and 80% ethanol for 10

**Table 1.** The primers used in qRT-PCR.

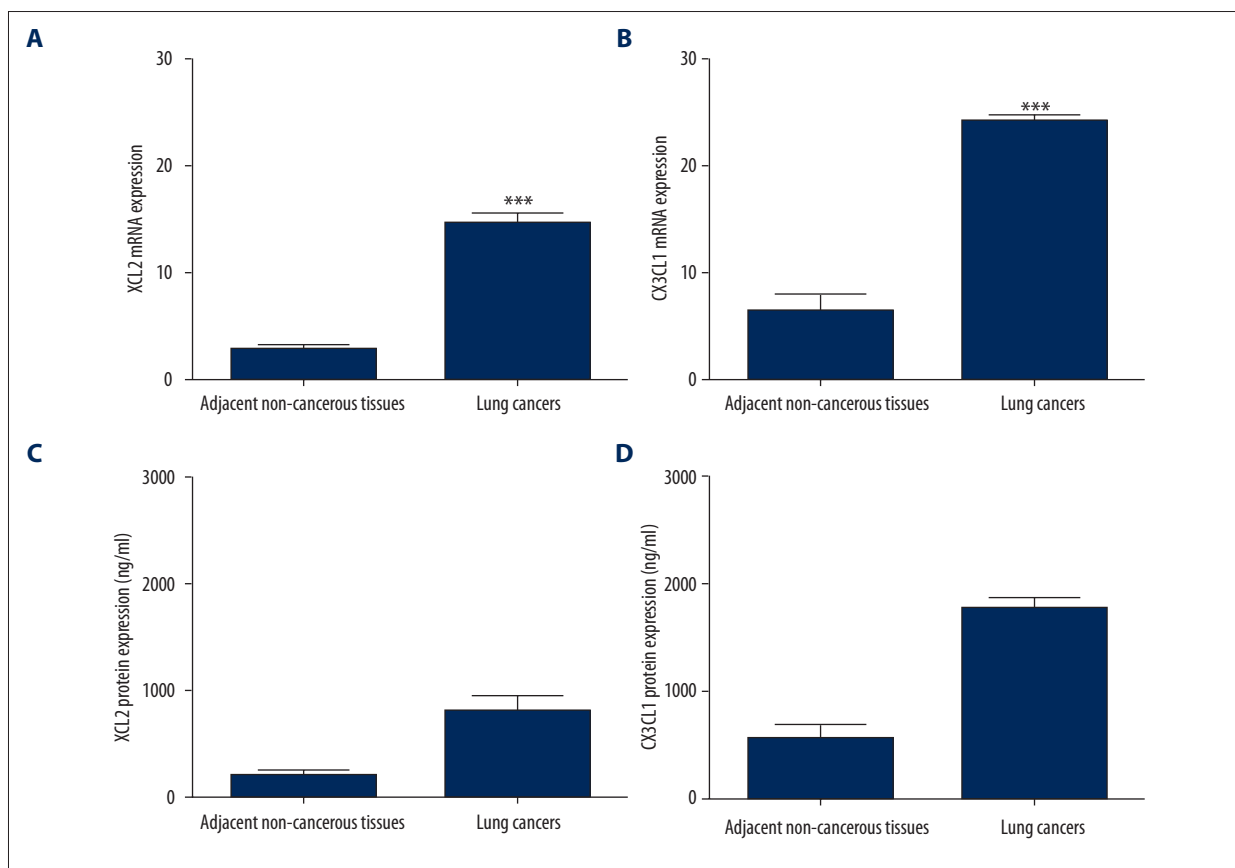
Gene	Primer (5'-3')	bp
XCL2	For: CAGTGCGTGTGCTGGAGT	158
	Rev: GGGTGAGAAGCTGAATCCA	
CX3CL1	For: ACTAAGAAAGACCCGAGGC	126
	Rev: GGGGCACGTTGGTGTTCAC	
GADPH	For: AGCCACATCGCTCAGACA	114
	Rev: TGGACTCCACGACGTACT	

min each. Sections were rinsed with water and subjected to antigen retrieval. The sections were blocked with horse serum and incubated with appropriate primary antibodies. The sections were then incubated with secondary antibodies, stained with hematoxylin solution, soaked in 1% hydrochloric acid alcohol, rinsed with running water anti-blue, dehydrated with an ethanol gradient, treated with xylene for transparency, and

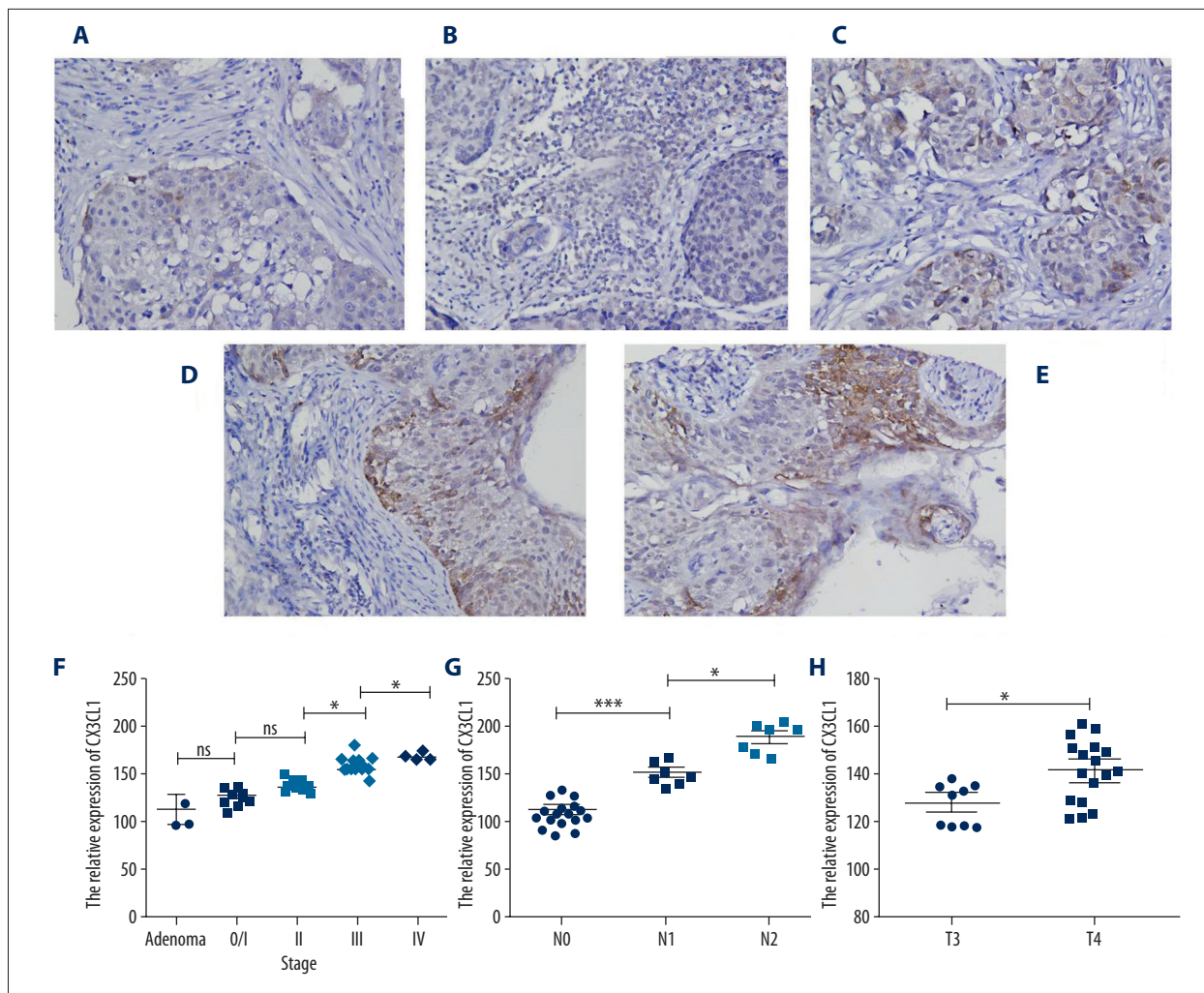
mounted with neutral gum. The sections were observed under a microscope. Cytoplasm of XCL2- and CX3CL1-positive cells were positively stained (colored yellow or brown). The positive rate was calculated as the ratio of the number of positive cells to the area of visual fields using Image J software, and compared to determine the difference in the expression level of both proteins.

**ELISA**

Human XCL2 and CX3CL1 ELISA reagents were purchased from Ray Biotech., Ltd. (Guangzhou, China). Total protein was extracted from lung cancer and normal tissues. Aliquots of 100 µL of samples were added to a 96-well plate. After 90 min of reaction at 37°C, 100 µL of biotinylated antibody was added to each well and allowed to react at 37°C for 60 min. The plate was washed 3 times with 0.01 M TBS. A total of 100 µL of ABC was added to each well. After 30 min of reaction at 37°C, the plate was rinsed 5 times with 0.01 M TBS. The plate was incubated with TMB at 37°C in the dark and the reaction was terminated by adding TMB stop solution after 30 min. The plate was detected using a microplate reader under 470 nm.



**Figure 1.** The expression of XCL2 and CX3CL1 mRNAs and proteins in lung cancer. The expression of XCL2 and CX3CL1 mRNAs and proteins in lung cancer was significantly increased compared with normal tissues. \*\*\* indicates P<0.001.



**Figure 2.** Immunohistochemical analyses showing stronger XCL2 expression in lung cancer in higher pathologic stages. (A–E) Immunohistochemical staining of lung cancers in different pathologic stages ranging from adenoma to stage IV cancer. (F) Comparison of relative XCL2 expression in lung cancer in different pathologic stages. (G) Comparison of relative XCL2 expression in lung cancer with different number of metastatic lymph nodes. (H) Comparison of relative XCL2 expression between T3 and T4 lung cancer. \* Indicates  $P < 0.05$ , and \*\*\* indicates  $P < 0.001$ .

### Statistical analysis

All tests were repeated at least 3 times and are presented as mean  $\pm$  standard deviation. Data were analyzed using SPSS 17.0. Difference between groups was compared by *t* tests. *P* values smaller than 0.05 were considered statistically significant.

## Results

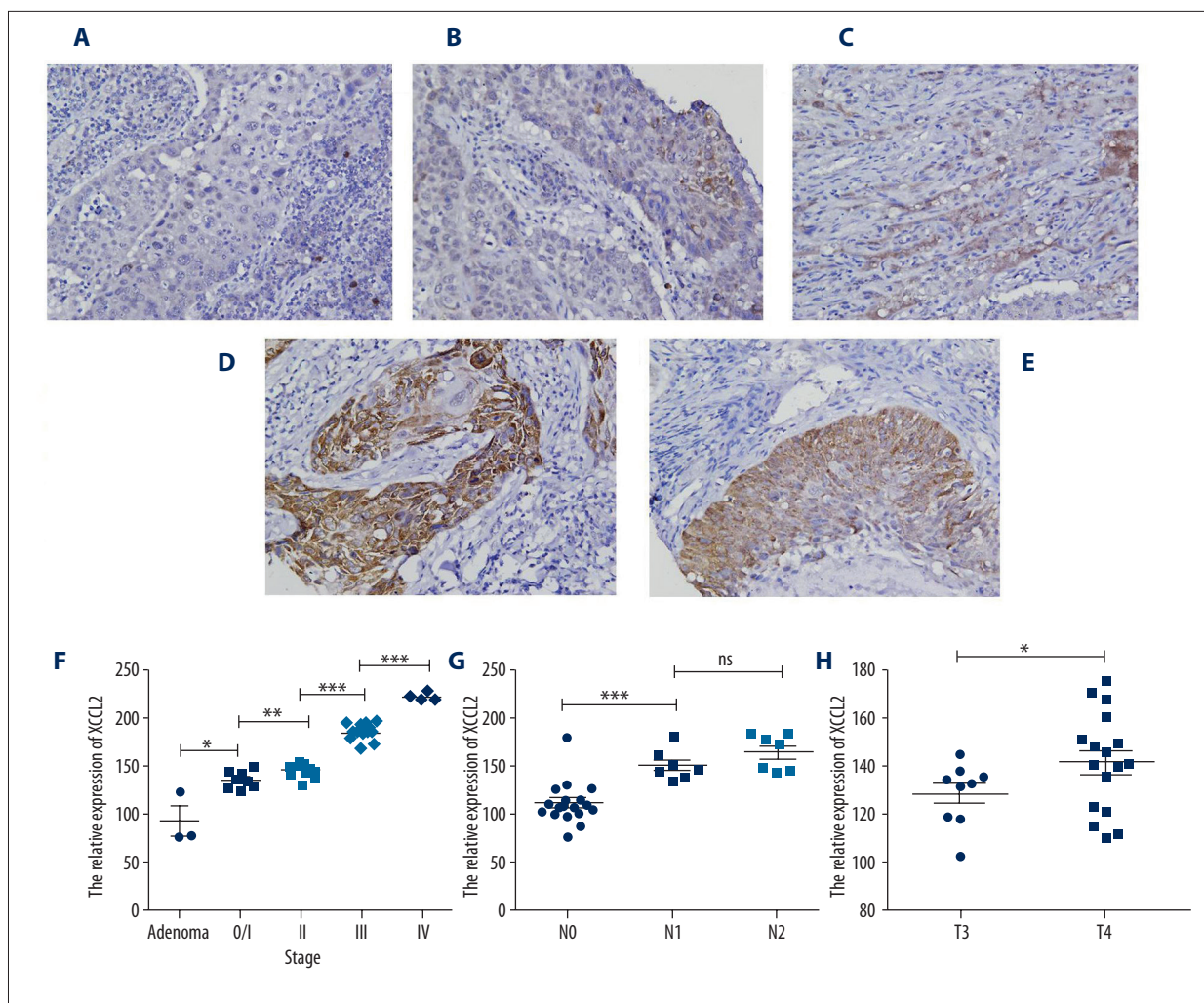
### XCL2 and CX3CL1 expression in lung cancer

As shown in Figure 1A and 1B, qRT-PCR analyses demonstrated that the expression of XCL2 and CX3CL1 mRNA in lung cancer was significantly higher than that in normal tissues ( $P < 0.05$ ).

The expression of XCL2 and CX3CL1 proteins was detected by ELISA. It was shown that the expression of both chemokines in lung cancer was also significantly increased compared with normal tissues ( $P < 0.05$ , Figure 1C, 1D).

### Comparison of XCL2 expression in lung cancers in different pathologic stages

XCL2 expression in lung cancers in different pathological stages was compared by immunohistochemical staining. It was shown that the expression level of XCL2 was significantly increased with higher pathological stages ( $P < 0.05$  or  $P < 0.001$ , Figure 2A–2F). XCL2 expression in cancers with higher numbers of metastatic lymph nodes was also significantly increased compared with cancers with lower numbers of metastatic lymph



**Figure 3.** Immunohistochemical analyses showing stronger CX3CL1 expression in lung cancer in higher pathologic stages. (A–E) Immunohistochemical staining of lung cancers in different pathologic stages, ranging from adenoma to stage IV cancer. (F) Comparison of relative XCL2 expression in lung cancer in different pathologic stages. (G) Comparison of relative XCL2 expression in lung cancers with different numbers of metastatic lymph nodes. (H) Comparison of relative XCL2 expression between T3 and T4 lung cancer. \* Indicates  $P < 0.05$ , and \*\*\* indicates  $P < 0.001$ .

nodes ( $P < 0.05$  or  $P < 0.001$ , Figure 2G). Moreover, XCL2 expression in T4 lung cancer was significantly higher compared with T3 cancer ( $P < 0.05$ , Figure 2H).

### Comparison of CX3CL1 expression in lung cancers in different pathological stages

Results of immunohistochemical analyses showed that CX3CL1 expression was significantly increased with increasing pathological stages ( $P < 0.05$ , Figure 3A–3F). CX3CL1 expression in N1 lung cancer was also significantly higher compared with N0 cancer ( $P < 0.001$ , Figure 3G). Moreover, CX3CL1 expression in T4 lung cancer was significantly higher compared with T3 cancer ( $P < 0.05$ , Figure 3H).

### Discussion

The occurrence and development of lung cancer is a multi-step, multi-factor process involving the deactivation of tumor suppressor genes and activation of oncogenes. Numerous studies have shown that chemokines play important regulatory roles in the pathogenesis of a variety of tumors, as well as in the proliferation, apoptosis, and signal transduction of tumor cells by acting as either tumor suppressors or promoters [10,11]. Differential expression of chemokines has been observed in different types of cancers. Wang et al. demonstrated that serum chemokine levels in patients with NSCLC are higher compared with healthy controls [12]. Jia et al. reported that chemokine concentration in NSCLC patients was lower than in patients with benign lung cancer [13]. Wu et al. found that chemokine

expression in NSCLC is associated with TNM staging, lymph node metastasis, survival rate, and overall response rate [14]. It has also been shown that the expression of chemokines is closely related to TNM staging and distant metastasis of NSCLC [15]. These findings suggest that the changes in chemokine expression are strongly associated with the pathological stages of lung cancer. Studies have also suggested the important prognostic value of chemokine expression for lung cancer [16].

Chemokines play important roles in the occurrence and development of cancers due to their chemotactic effect on cancer cells [17]. Studies have demonstrated that CCR1, the CCL5 receptor, and CX3CL1 synergistically induce liver metastases from colorectal cancer [18]. The regulation of chemokines is crucial for the proliferation and apoptosis of tumor cells. High expression of XCL2 has been reported in a variety of cancers, including melanoma, NSCLC, prostate cancer, and colorectal cancer [19]. In our study, the expression of XCL2 mRNA and protein in lung cancer was significantly higher compared with normal tissues. Consistent with our results, Kamimura et al. found that XCL2 is overexpressed in lung cancer and is associated with the prognosis of the disease [20]. Liu et al. demonstrated that CX3CL1 expression is gradually increased in benign lung tissue, primary lung tumors, and lymph node metastases, and that the overall survival rate of patients with stronger CX3CL1

expression is much lower compared with those with weaker CX3CL1 expression [21]. In a study of CX3CL1 expression in NSCLC, Howard et al. demonstrated that CX3CL1 expression in cancer tissues is markedly higher than that in adjacent normal tissues [22]. It has also been shown that serum CX3CL1 expression is positively correlated with both serum VEGF expression and microvessel density in tumors, suggesting that CX3CL1 overexpression is closely associated with the occurrence and development of lung cancer [23]. In our study, CX3CL1 expression was stronger with increasingly higher pathological stages.

In summary, the current study demonstrated that XCL2 and CX3CL1 expression in lung cancers were significantly higher compared to adjacent normal tissues. Moreover, expression of both chemokines was significantly stronger with higher pathological stages. We speculate that XCL2 and CX3CL1 synergistically promote the development of lung cancer.

## Conclusions

Our findings indicate that both chemokines might be important targets in gene therapy for lung cancer, and their antagonists might have an anti-lung cancer effect. However, the relevant mechanism needs to be investigated in further studies.

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