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RESEARCH ARTICLE

Clinical significance of expression level of CX3CL1–CX3CR1 axis in bone metastasis of lung cancer

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Abstract

Purpose To investigate the clinical significance of CX3 chemokine ligand 1(CX3CL1) and CX3CR1 in patients with bone metastasis from lung cancer. The expression levels of CX3CL1 and CX3CR1 mRNA and protein in primary lung cancer and lung cancer bone metastasis were detected by qRT-PCR and Western blot.

Methods One hundred patients with lung cancer were divided into a boneless metastasis group (50 patients with bone metastasis) and a bone metastasis group (50 patients without distant metastasis). The bone transfer component was graded by Soloway classification (0 to III). The expression levels of serum CX3CL1–CX3CR1 axis were detected by enzyme-linked immunosorbent assay (ELISA). RT-qPCR and Western Blot were used to verify the transfection efficiency. The scratching assay was used to detect the migration of CX3CL1 to 95-D cells after down-regulating the expression of CX3CR1.

Results The expression levels of CX3CL1 and CX3CR1 mRNA and protein in the primary lung cancer and lung cancer bone metastasis were significantly higher than those in the adjacent tissues ($P < 0.0001$). The levels of serum CX3CL1 and CX3CR1 in bone metastasis group were significantly higher than those in boneless metastasis group and healthy control group ($P < 0.05$). In the bone metastasis group, the levels of serum CX3CL1 and CX3CR1 were significantly positively correlated with the degree of disease progression ($P < 0.01$).

Conclusion The expression level of serum CX3CL1–CX3CR1 axis is expected to be an auxiliary reference index for monitoring bone metastasis of lung cancer.

Keywords CX3CL1–CX3CR1 axis · Lung cancer · Bone metastasis · Lung cancer

Introduction

Lung cancer is one of cancers in the world with the highest incidence and mortality. Its incidence is hidden, and most of them are advanced at the time of diagnosis. Distant metastasis, especially bone metastasis, is an important cause of death in patients with lung cancer. Bone metastasis can cause various types of bone disease and even paralysis in patients with lung cancer, which seriously reduces the quality of life of patients [1, 2]. Once bone metastasis occurs, the survival rate of patients with lung cancer will also be greatly reduced. As the incidence of bone metastasis in lung cancer is relatively elusive, there is a lack of effective early detection methods, thus it is easy to miss the best expectations, and patients often have different degrees of metastasis when diagnosed. Therefore, early identification and detection of bone metastasis from lung cancer can help early treatment, improving the quality of patients' life [3, 4]. At present, conventional testing methods cannot detect

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bone metastasis from cancer in time, and some sensitive tests have defects such as radiation damage, cumbersome operation, and high cost. Therefore, new methods are needed for early diagnosis and treatment of lung cancer bone metastasis [5, 6]. The detection of serum tumor markers is safe with high efficiency, simple operation, and low price, which plays an important role in the clinical diagnosis and treatment of cancer [7, 8]. Chemokine is a class of secretory protein superfamily, which has chemotaxis and can regulate the adhesion and migration of leukocytes, lymphocytes and monocytes. At present, more than 40 chemokines are known, and they were divided into four categories: CXC, CC, C, and CX3C, of which CX3CL1 is located on human chromosome 16q13, which is the only member of the chemokine CX3C family. CX3CR1 is the only receptor for CX3CL1. Studies have found that Cx3CLl and its receptors are expressed in many different types of malignant tumors, and chemokines are malignant, which play an important role in tumor proliferation, invasion, and migration [9, 10]. Studies have also found that the expression level of CX3CL1–CX3CR1 axis is related to spinal metastasis in a variety of cancers [11]. Studies in breast cancer have found that the expression of CX3CR1 was increased in breast cancer with brain metastasis [12]. In the study of prostate cancer, it was found that CX3CR1 is involved in bone metastasis of prostate cancer, and bone epithelial cells secrete the chemokine CX3CLl [10]. At present, there is no report on the role of the expression of CX3CL1–CX3CR1 axis in bone metastasis of lung cancer. Studies found that the expression of CX3CL1 in the serum of patients with spinal metastasis of lung cancer was higher than that of the normal control group, suggesting that CX3CL1 may participate in spinal metastasis of lung cancer and play a role in this process with important promoting effect [13]. This study examined the expression levels of serum CX3CL1 and CX3CR1 in 100 patients with lung cancer, and preliminary explored the clinical value of the expression levels of serum CX3CL1–CX3CR1 axis on bone metastasis of lung cancer. At the same time, the effect of CX3CL1/CX3CR1 axis on the metastasis ability of human lung cancer cell line 95-D was studied at the cell level, so as to provide a new basis for the diagnosis of bone metastasis of lung cancer.

Materials and methods

Clinical data and cells

A total of 100 patients with lung cancer in our hospital from January 2016 to January 2018 were collected and the surgically resected specimens were stored at − 80° C. At the initial diagnosis, they were divided into two groups, including 50 cases in the bone metastasis group and 50 cases in the non-bone metastasis group. Patients in the non-bone metastasis group were followed up, and 15 new cases with lung cancer bone metastasis were found after half a year. Another 40 healthy people who underwent a physical examination at the same period were selected as the healthy control group, including 27 males and 13 females, 44–68 years, with an average age of (55 ± 2.6) years. The cancer tissues, corresponding normal tissues of patients with primary lung cancer, and the bone metastases of lung cancer were collected and stored at − 80 °C. All study subjects signed informed consent.

Lung cancer bone metastasis diagnostic criteria: patients with bone disease symptoms such as bone pain; diagnosed to be bone metastasis examined by MRI or CT or whole body bone scan or X-ray. All subjects included did not receive chemoradiotherapy and immunotherapy. Based on Soloway classification standard [3], the bone metastasis was divided into 4 grades according to the degree of bone metastasis, the number of main lesions and bone imaging of lung cancer. Grade 0 means bone imaging lesions are benign. Grade I means there are 1 to 2 places with bone metastasis. Grade II means there are 3 to 5 places with bone metastasis. Grade III means there are more than 5 places with bone metastasis. According to this classification, 50 patients in the lung cancer bone metastasis group were divided into three groups (25 cases in the Grade I group, 18 cases in the Grade II group, 7 cases in Grade III group).

Exclusion criteria: (1) exclusion criteria for the bone metastasis group: patients with severe heart, liver, and kidney disease; patients with traumatic fractures; patients with osteoporosis and bone metabolic diseases caused by other diseases; patients with malignant tumors in other parts; (2) exclusion criteria for non-bone metastasis group: patients with severe heart, kidney, and liver diseases.

Human lung cancer cells (lymph node metastasis) NCI-H292, human high-metastasis lung cancer cells 95-D, human high-metastasis lung cancer cell line PG cells, human high-metastasis lung cancer cells PGCl3, and human normal bronchial epithelial cells HBE were purchased from the cell bank of the Chinese Academy of Sciences.

Bone scan and CT examination

GE Discovery NM/CT 670 machine from the USA was used to analyze the results by more than 2 physicians. The radioactivity was abnormally increased or decreased compared with the adjacent and contralateral bone tissue, and it was diagnosed as positive when other bone lesions were excluded. When the abnormal focus is suspicious, a CT scan was performed to further confirm the diagnosis.

qRT-PCR

Tissue RNA was extracted, cDNA was synthesized by reverse transcription, and frozen at − 20 °C. Primer 5.0 was used to design CX3CL1 and CX3CR1 primers, CX3CL1 upstream primer was 5′-GTCATCTATACAATGTTACC-3′, downstream primer was 5′-GTGTTAGGCTTCTGGGCA C-3′. CX3CR1 upstream primer was 5′-GGTAGCTGCACG TTGTCCGC-3′, downstream primer was 5′-CAGCGAGCA CCTTGGTGTAC-3′. qRT-PCR total reaction system was 25.0 μL, the reaction conditions of CX3CL1 were: 95 °C for 30 s, 57 °C for 35 s, 72 °C for 25 s, for 45 cycles, and the reaction conditions of CX3CR1 were: 95 °C for 35 s, 55 °C for 35 s, and 72 °C for 30 s, for 45 cycles. The reaction products were subjected to 1.5% agarose gel electrophoresis, photographed, and gray values were analyzed by Image Lab software.

Western blot

The tissues of each group were collected, lysed, centrifuged, and the supernatant was collected. The protein concentration was determined by the BCA method. Fifty ug of protein was mixed with the loading buffer (5 X) in a ratio of 4: 1, denatured in a boiling water bath, and cooled to load. SDS-PAGE was used to coagulate Gel electrophoresis. The membrane is transfer at room temperature in the shaker, with 30 mL of TBST, 1.5 g of skim milk powder was equipped with 5% sealing liquid for 2 h. Primary antibody was CX3CL1 1:1000, CX3CR1 1:1500, and β-actin 1:2000. PVDF membrane was put into secondary antibody (1:5000), incubated at room temperature for 1 h. The membrane was washed, developed, fixed, and the gray value was analyzed by the Quantity One software.

Determination of serum CX3CL1 and CX3CL1 levels

The venous blood was collected, and the serum was collected by centrifugation, and stored at − 80 °C. A fully automatic microplate reader (ML-STAR venus CH.8, Swiss Ausbon Biological Engineering Co., Ltd.) was used, and the serum CX3CL1 (Nanjing Jitai Biological Company, SBJ-H0655) and CX3CR1 (Shanghai Jima Biotechnology Co., Ltd., YM-QP12286) were detected by ELISA.

Transient transfection

95-D cells in the logarithmic growth phase were seeded in a six-well plate (6×10^4 cells/well), and the cells were plated at a density of about 50%. The transfection group was added with Lifectamine2000 and CX3CR1-shRNA plasmids for 4 h. The medium was used to continue to be cultured, and observed and photographed under a fluorescent inverted microscope. The control group was normal culture cells. There are four groups: (1) blank control group (Con-A group), cells without any treatment; (2) no-load control group (Con-B group), cells treated with transfection reagent only; (3) negative control group (Con-A group), cell transfection disrupted or non-silent plasmid sh-NC; (4) In the sh-CX3CR1 group, cells were transfected with sh-CX3CR1.

Cell scratch test

The cells in logarithmic growth phase were seeded in a six-well plate. When the cells were adherently grown to a density plating area of about 90%, they were scratched with a 10 μL pipette tip, with three replicates in each group. And the cells were washed with PBS and basal medium was added to continue to culture, observed and taken pictures under the microscope at 0 h and 24 h, the width of the scratch was measured and the scratch healing rate was calculated.

Statistical methods

Analysis was performed using SPSS 20.0 software. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$); differences between the two groups were tested by t test, comparisons between multiple groups were analyzed by variance. Non-parametric samples were analyzed using Wilcoxon paired test. Correlation analysis was performed by non-parametric Spearman correlation analysis. $P<0.05$ indicates that the difference was statistically significant.

Results

CX3CL1 and CX3CR1 are highly expressed in lung cancer and bone metastasis of lung cancer

After qRT-PCR and Western blot detection, it was found that the expression levels of CX3CL1mRNA and protein in primary lung cancer and lung cancer bone metastasis tissues were significantly higher than those in adjacent tissues, and the expression of CX3CL1mRNA and protein levels in lung cancer bone metastasis tissues were higher than that in primary lung cancer. The expression levels were higher than those in the original lung cancer tissues, and the differences were statistically significant (both of them in Fig. 1a, b were $P<0.0001$). The expression levels of CX3CR1mRNA and protein in the original lung cancer and bone metastasis tissues were higher than that in paracancerous tissues. The expression in bone metastatic tissue of lung cancer was significantly higher than that in primary lung cancer tissue, and the differences were statistically significant (all of them in Fig. 1c, d were $P<0.0001$). Spearman

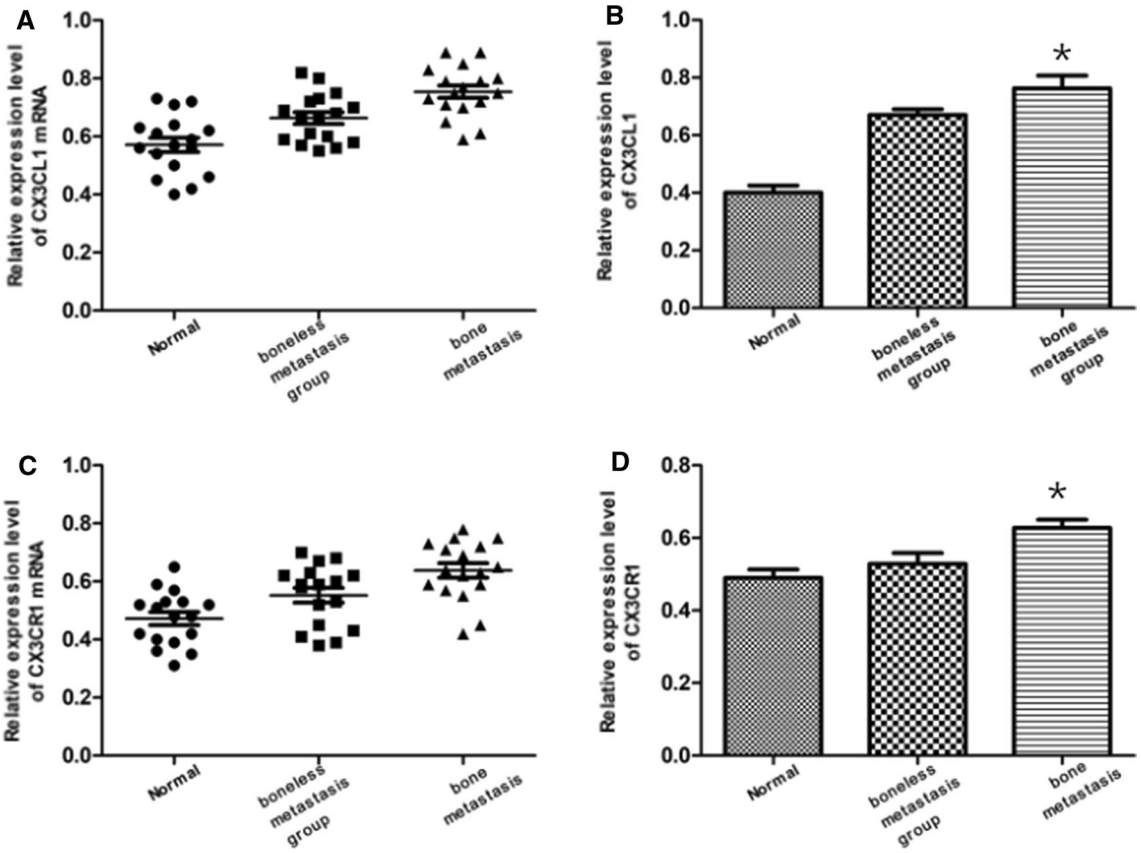


Fig. 1 **a** qRT-PCR detection of CX3CL1mRNA expression in primary lung cancer, lung cancer bone metastasis and adjacent tissues. It was found that the expression level of CX3CL1mRNA was: lung cancer bone metastasis tissue > primary lung cancer tissue > paraneoplastic tissue. Statistical analysis of CX3CL1mRNA expression in primary lung cancer, lung cancer bone metastasis and adjacent tissue. **b** Western blot analysis of the expression of CX3CL1 protein in primary lung cancer, lung cancer bone metastases, and adjacent tissues. It was found that the expression level of CX3CL1 protein was: lung cancer bone metastasis tissue > primary lung cancer tissue > paraneoplastic tissue. **c** qRT-PCR detection of the CX3CR1mRNA expression in primary lung cancer and lung cancer bone metastasis and adjacent tissues. It was found that the expression level of CX3CR1mRNA was: lung cancer bone metastasis tissue > primary lung cancer tissue > paraneoplastic. **d** Western blot analysis of CX3CR1 protein expression in primary lung cancer and lung cancer bone metastasis and adjacent tissues. It was found that the expression level of CX3CR1 protein was: lung cancer bone metastasis tissue > primary lung cancer tissue > paraneoplastic tissue. The experiment was repeated 3 times. *$P < 0.055$

correlation Analysis showed that the expression of CX3CL1 and CX3CR1 proteins in lung cancer tissues was positively correlated ($P < 0.001$), suggesting that the expression of CX3CL1–CX3CR1 axis may play a role in promoting lung cancer growth and bone metastasis.

Relationship between the expression of CX3CL1 and CX3CR1 in primary lung cancer and bone metastasis from lung cancer and its relationship with clinicopathological parameters

The relationship between the expression of CX3CL1 and CX3CR1 in primary lung cancer and bone metastasis from lung cancer and the clinicopathological parameters are shown in Table 1. According to the median value of CX3CL1 expression in 100 patients with lung cancer, the patients were divided into two groups: CX3CL1 high expression group (> median value) and CX3CL1 low expression group (< median value), and the patients were divided into CX3CR1 high expression group (> median value) and CX3CR1 low expression group (< median value) on the basis of the median value of CX3CR1 expression. The results showed that the expression of CX3CL1 had no relationship with the gender, age, and degree of invasion of patients with lung cancer, and the tumor diameter, degree of differentiation, Duke stage, and lymph node metastasis rate of patients with high expression of CX3CL1 were higher than those of patients with low expression of CX3CL1. The expression of CX3CR1 had no relationship with the gender, age, and degree of invasion of patients with lung cancer. The tumor diameter, differentiation degree, Duke stage, and lymph node metastasis rate were higher in the high

Table 1 Relationship between the expression of CX3CL1 and CX3CR1 and clinicopathological parameters of 100 cases with lung cancer

Pathological parameters	*n*	CX3CL1 expression		*P* value	CX3CR1 expression		*P*-value
		Low expression	High expression		Low expression	High expression	
Age (years)				0.764			0.635
> 65	52	29	23		32	20	
≤ 65	48	36	12		18	30	
Gender				0.457			0.596
Male	39	16	23		15	24	
Female	61	27	34		51	10	
Tumor diameter (cm)				0.103			0.203
> 1.32	37	19	18		12	25	
≤ 1.32	63	35	28		50	13	
Bone metastasis				0.002			0.005
No	50	23	27		15	35	
Yes	50	16	34		19	31	
Infiltration depth				0.305			0.632
T_1	10	4	6		3	7	
T_2	25	12	13		11	14	
T_3	43	25	18		23	20	
T_4	22	16	6		12	10	
Differentiation				0.035			0.026
High	26	6	20		3	23	
Middle	49	14	35		19	30	
Low	25	11	14		6	19	
Duke staging				0.027			0.015
A	24	9	15		4	20	
B	23	6	17		5	18	
C	39	15	18		19	20	
D	14	4	10		3	11	

expression of CX3CR1 group than those in the CX3CR1 low-expression group.

Analysis of the levels of serum CX3CL1 and CX3CR1 in patients with lung cancer, lung adenocarcinoma and lung squamous cell carcinoma

Among 100 patients with lung cancer, there were 50 cases in the bone metastasis group, 50 cases in the non-bone metastasis group, 55 cases with adenocarcinoma and 45 cases with squamous cell carcinoma. The levels of serum CX3CL1 and CX3CR1 between patients with different age, sex, primary focus and pathological type of lung cancer were not statistically significant ($P < 0.05$). The expression of CX3CL1 and CX3CR1 in lung cancer patients with bone metastasis was higher than that in patients without bone metastasis (both were $P < 0.05$). See Table 2.

Among the 55 patients with lung adenocarcinoma, there were 23 in the bone metastasis group and 32 in the non-bone metastasis group. There was no statistically significant

Table 2 Comparison of clinical data and the levels of serum CX3CL1 and CX3CR1 of 100 patients with lung cancer (ng/mL, $x \pm s$)

Factor	*n*	CX3CL1	CX3CR1
Age (years)			
> 65	52	0.675 ± 0.102	0.658 ± 0.008
≤ 65	48	0.664 ± 0.047	0.635 ± 0.011
Gender			
Male	39	0.632 ± 0.016	0.665 ± 0.017
Female	61	0.635 ± 0.023	0.637 ± 0.061
Pathological type			
Adenocarcinoma	55	0.654 ± 0.041	0.680 ± 0.124
Squamous cell carcinoma	45	0.693 ± 0.197	0.604 ± 0.018
Transfer situation			
Bone metastasis	50	0.763 ± 0.014*	0.706 ± 0.016*
Boneless metastasis	50	0.694 ± 0.112	0.692 ± 0.027
Primary foci diameter (cm)			
> 1.32	37	0.669 ± 0.142	0.687 ± 0.023
≤ 1.32	63	0.632 ± 0.175	0.665 ± 0.164

Compared with the non-bone metastasis group, *$P < 0.05$

Table 3 Comparison of clinical data and the levels of serum CX3CL1 and CX3CR1 in 55 patients with lung adenocarcinoma (ng/mL, $x \pm s$)

Factor	*n*	CX3CL1	CX3CR1
Age (years)			
> 65	34	0.639 ± 0.013	0.636 ± 0.009
≤ 65	21	0.625 ± 0.009	0.672 ± 0.016
Gender			
Male	19	0.636 ± 0.014	0.663 ± 0.014
Female	36	0.687 ± 0.018	0.699 ± 0.024
Transfer situation			
Bone metastasis	23	0.765 ± 0.011*	0.706 ± 0.015*
Boneless metastasis	32	0.654 ± 0.008	0.726 ± 0.024
Primary foci diameter (cm)			
> 1.35	38	0.693 ± 0.013	0.638 ± 0.027
≤ 1.35	17	0.663 ± 0.021	0.657 ± 0.031

Compared with the non-bone metastasis group, *$P < 0.05$

Table 4 Comparison of clinical data and the levels of serum CX3CL1 and CX3CR1 in 45 patients with lung squamous cell carcinoma (ng/mL, $x \pm s$)

Factor	*n*	CX3CL1	CX3CR1
Age (years)			
> 65	18	0.687 ± 0.001	0.715 ± 0.003
≤ 65	27	0.674 ± 0.001	0.707 ± 0.003
Gender			
Male	27	0.635 ± 0.024	0.596 ± 0.005
Female	18	0.639 ± 0.005	0.681 ± 0.141
Transfer situation			
Bone metastasis	21	0.653 ± 0.007*	0.708 ± 0.005*
Boneless metastasis	24	0.636 ± 0.021	0.625 ± 0.002
Primary foci diameter (cm)			
> 1.30	38	0.674 ± 0.014	0.687 ± 0.005
≤ 1.30	17	0.638 ± 0.004	0.652 ± 0.018

Compared with the non-bone metastasis group, *$P < 0.05$

difference in the levels of serum CX3CL1 and CX3CR1 among patients with lung adenocarcinoma with different ages, genders, primary tumor sizes, and pathological types ($P < 0.05$). The levels of serum CX3CL1 and CX3CR1 in patients with bone metastasis in lung adenocarcinoma were higher than those in patients without bone metastasis (both were $P < 0.05$). See Table 3.

Among the 45 patients with lung squamous cell carcinoma, there were 21 cases in the bone metastasis group and 24 cases in the non-bone metastasis group. There was no statistically significant difference in the levels of serum CX3CL1 and CX3CR1 among patients with lung squamous cell carcinoma with different ages, genders, primary tumor sizes, and pathological types ($P < 0.05$). The levels of serum CX3CL1 and CX3CR1 in patients with bone metastasis in lung squamous cell carcinoma were higher than those without bone metastasis (both were $P < 0.05$). See Table 4.

Comparison of the levels of serum CX3CL1 and CX3CR1 in each group

The levels of serum CX3CL1 and CX3CR1 in each group of bone metastasis group from Grade I to Grade III lung cancer were significantly higher than those of non-bone metastasis group and healthy control group, the difference was statistically significant (all were $P < 0.01$). The levels of serum CX3CL1 and CX3CR1 in each group of bone metastasis group from Grade I to Grade III lung cancer were statistically significant (both were $P < 0.01$). There was no statistically significant difference in the levels of serum CX3CL1 and CX3CR1 in the non-bone metastasis group compared with that in the healthy control group ($P = 0.563$ and 0.681). See Table 5.

Correlation between the levels of serum CX3CL1 and CX3CR1 and bone metastasis in different grades of lung cancer

The levels of serum CX3CL1 were significantly positively correlated with lung cancer bone metastasis ($r = 0.758$, $P < 0.01$), the levels of serum CX3CR1 were significantly positively correlated with lung cancer bone metastasis ($r = 0.701$, $P < 0.01$); serum CX3CL1 and CX3CR1 were significantly positively correlated ($r = 0.799$, $P < 0.01$). In the Grade I to Grade III bone metastasis group, the Levels of Serum CX3CL1 and CX3CR1 gradually increased with the increase of bone metastasis grades, and the levels of serum CX3CL1 and CX3CR1 were significantly positively correlated with disease progression ($P < 0.01$). See Table 6.

Elevated the levels of serum CX3CL1 and CX3CR1 predict the new bone metastasis in patients with lung cancer

50 patients with non-bone metastasis lung cancer were followed up. 6 months later, 16 new cases with bone metastasis lung cancer appeared. The levels of serum CX3CL1 and CX3CR1 in the newly diagnosed lung cancer bone metastasis group and new-onset lung cancer bone metastasis cancer group were significantly higher than those in non-bone metastasis group. The difference was statistically significant (both were $P < 0.05$). The levels of serum CX3CL1 and CX3CR1 in the newly diagnosed lung cancer bone metastasis group were higher than those in the new-onset diagnosed lung cancer bone metastasis group, and the difference was not statistically significant ($P > 0.05$, Table 7).

Table 5 Comparison of the levels of serum CX3CL1 and CX3CR1 in each group

Group	*n*	CX3CL1(ng/mL)	P_1	CX3CR1(ng/mL)	P_2
Healthy control group	40	0.633 ± 0.03	–	0.621 ± 0.16	–
Boneless metastasis group	50	0.637 ± 0.06	0.563	0.659 ± 0.62	0.681
Bone metastasis with lung cancer group					
Grade I	25	0.665 ± 0.14	0.000	0.688 ± 0.05	0.008
Grade II	18	0.695 ± 0.20	0.006	0.692 ± 0.02	0.000
Grade III	7	0.793 ± 0.16	0.003	0.791 ± 0.01	0.000

Table 6 ROC curve area (AUC) and correlation of serum CX3CL1 and CX3CR1 in diagnosis of bone metastasis in bone metastasis group

Bone metastasis	*n*	CX3CL1		CX3CR1	
		AUC	OR	AUC	OR
Grade I	25	0.905	0.602	0.912	0.729
Grade II	18	0.938	0.718	0.947	0.780
Grade III	7	0.979	0.894	0.982	0.863

Expression of CX3CR1 in common human metastatic lung cancer cells

Detection of CX3CR1 mRNA and protein expression levels in four common human high-metastasis lung cancer cells, human lung cancer cells (lymph node metastasis) NCI-H292, human high-metastasis lung cancer cells 95-D, human high-metastasis lung cancer cell line PG cells and human high-metastasis lung cancer cells PGCl3, and human normal bronchus epithelial HBE. RT-PCR and Western Blot experiments showed that the mRNA and protein expression of CX3CR1 in 95-D cells were higher than those in other cells.

CX3CL1 promotes migration of human lung cancer cell line 95-D with high metastasis

The scratch test results showed that after treating cells with different concentrations (0, 50, 100, 200, 300 ng/mL) of CX3CL1 recombinant protein for 24 h, the scratch spacing decreased with the increase of CX3CL1 concentration ($P < 0.05$). It suggested that CX3CL1 recombination protein can up-regulate cell migration ability. See Fig. 2.

Table 7 Comparison of the levels of serum I CTP and BSP in newly diagnosed lung cancer bone metastasis group, bone metastasis free group and new-onset lung cancer bone metastasis group

Group	Number of cases	CX3CL1 (ng/mL)	P_1	CX3CR1 (ng/mL)	P_2
Boneless metastasis group	50	0.663 ± 0.02	–	0.679 ± 0.12	–
New bone metastasis group	16	0.694 ± 0.22	0.013	0.689 ± 0.04	0.003
Newly diagnosed bone metastasis group	50	0.703 ± 0.16	0.002	0.705 ± 0.17	0.019

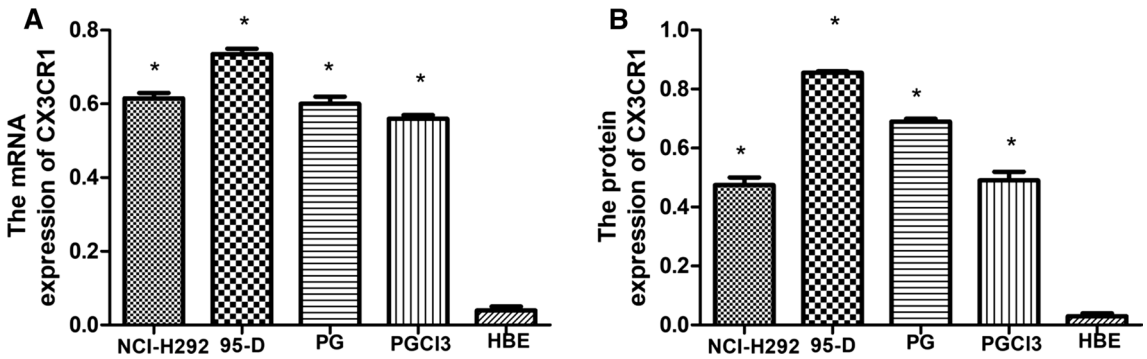


Fig. 2 CX3CR1 mRNA and protein expression levels in common human high-metastasis lung cancer cells. **a** RT-PCR test detected the expression level of CX3CR1 mRNA in NCI-H292, 95-D, PG, PGCl3, HBE. **b** Western Blot test detected the expression level of CX3CR1 protein in NCI-H292, 95-D, PG, PGCl3, HBE

CX3CL1 depends on its receptor CX3CR1 affects 95-D migration ability of human lung cancer high metastatic cell lines

Western Blot experiments showed that the expression of CX3CR1 increased with the increase of CX3CL1 concentration, suggesting that CX3CL1 can promote the expression of CX3CR1 in 95-D cells (Fig. 3a).

sh-CX3CR1 was transfected with 95-D cells, and Western Blot was used to verify the transfection efficiency. The results showed that CX3CR1 expression was down-regulated (Fig. 3b). The healing rate of 100 ng/mL CX3CL1 cells increased compared with the Blank group (normally cultured cells). Cell scratch experiments were used to detect the effect of CX3CL1 on the migration capacity of 95-D cells after CX3CR1 expression was down-regulated. The results showed that down-regulating the expression of CX3CR1 reduced the effect of CX3CL1 on 95-D cell migration, suggesting that CX3CL1 depends on its receptor CX3CR1 to affect 95-D cell migration capacity (Fig. 3c).

CX3CR1 expression promoted by CX3CL1 in common human lung cancer high-metastasis cell lines

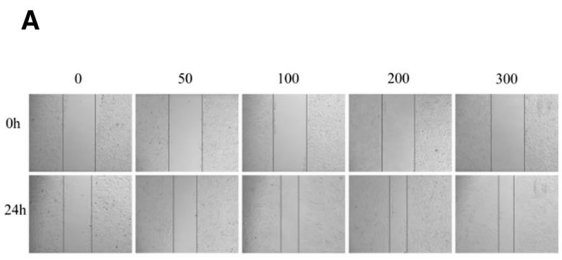
Western Blot experiments showed that in human lung cancer cells (lymph node metastasis) NCI-H292, human high-metastasis lung cancer cell line PG cells and human high-metastasis lung cancer cell PGCl3 cells, the expression of CX3CR1 increased first and then decreased with the increase of CX3CL1 concentration. In high metastatic lung cancer cells, CX3CL1 can promote the expression of CX3CR1 in a certain concentration range (Figs. 4, 5.

Discussion

Lung cancer is one of the malignant tumors with high morbidity and high mortality in the world. Patients with lung cancer are prone to have bone metastasis, different degrees of bone pain and dysfunction and various serious complications, leading to a decline in the quality of life of patients. At present, the diagnosis of bone metastasis of lung cancer mainly depends solely on some common imaging techniques. These methods have many limitations, such as low sensitivity, which can only be visualized when the bone damage reaches a certain severity; the equipment is expensive; the detection will cause radiation damage to the patient, etc. It is necessary to study some new methods for diagnosis of bone metastasis in lung cancer [14, 15].

In recent years, serological markers and some molecular indicators related to bone metabolism have gradually been applied to the diagnosis and treatment of bone metastasis, including bone metastasis in lung cancer. Studies have found that chemokines play an important role in tumor proliferation, invasion and other aspects. CX3CL1 has two forms: membrane-bound/secreted, which is a special type of chemokine, with the ability to mediate tumor-related inflammatory responses and the ability to quickly adhere to CX3CR1 expressing cells. CX3CR1 is the only receptor for CX3CL1, which is expressed in dendritic cells, NK cells, CD8 + T cells, and monocytes. Studies have shown that the CX3CL1–CX3CR1 axis is up-regulated in breast cancer, colon cancer, gastric cancer, prostate cancer and other malignancies. Tumor cells expressing CX3CL1 can promote the invasion and metastasis of CX3CR1-positive tumor cells [10, 16–18].

In this study, qRT-PCR and Western blot were used to detect the expression of CX3CL1 and CX3CR1 in 100



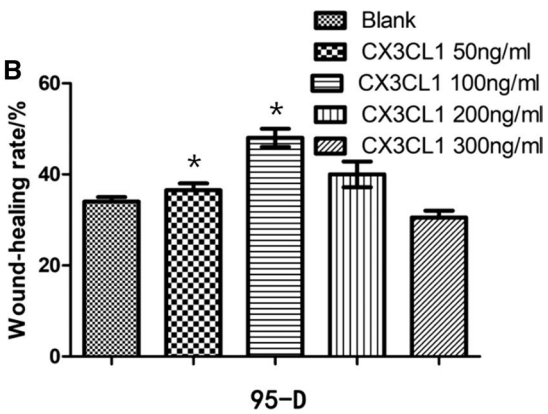


Fig. 3 Cell scratch test to detect the changes in cell migration ability of CX3CL1 recombinant protein treated cells at different concentrations (0, 50, 100, 200, 300 ng/mL) after treated for 24 h. **a** Cell scratch test. The scratch spacing decreases with the increase of CX3CL1 concentration (0, 50, 100 ng/mL). **b** Wound-healing rate. Wound-healing rate increases with increasing CX3CL1 concentration (0, 50, 100 ng/mL). When compared with the control group, $P < 0.05$ was statistically significant

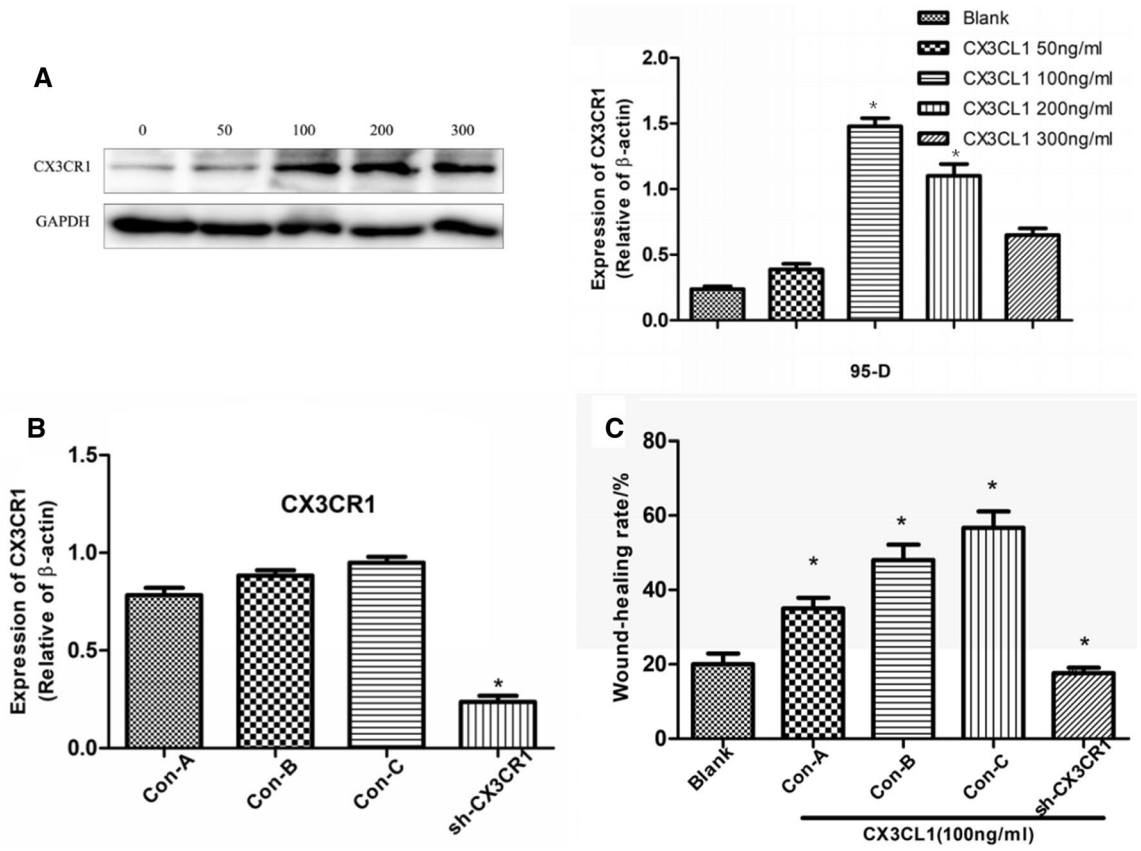


Fig. 4 CX3CL1 depends on its receptor CX3CR1 to affect the 95-D migration ability of high metastatic cell lines of human lung cancer. **a** Western Blot detected the changes of CX3CR1 expression after different concentrations of CX3CL1 treated 95-D cells. The expression of CX3CR1 increases with the increase of CX3CL1 concentration. **b** sh-CX3CR1 was transfected into 95-D cells, and Western Blot verified the transfection efficiency. CX3CR1 expression was down-regulated after transfection. Con-A group: blank control group, cells without any treatment; Con-B group: empty control group, only treated with transfection reagent; Con-C group: negative control group, cell transfection disturbed or non-silent plasmid sh-NC; sh-CX3CR1 group, cells transfected with sh-CX3CR1. **c** Scratch assay was used to detect the effect of CX3CL1 on the migration ability of 95-D cells after CX3CR1 expression was down-regulated. The healing rate of 100 ng/mL CX3CL1 cells was higher than that of the Blank group. Down-regulation of CX3CR1 expressing cells reduced the rate of healing. That is, down-regulation of CX3CR1 expression reduced the effect of CX3CL1 on 95-D cell migration. The experiment was repeated 3 times. $*P < 0.055$

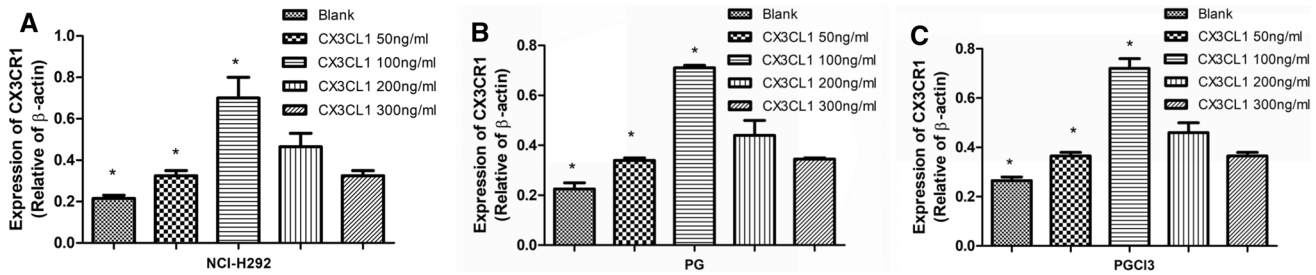


Fig. 5 Cell scratch test. After detecting different concentrations of CX3CL1 recombinant protein at different concentrations (0, 50, 100, 200, 300 ng/mL) for 24 h, the change of cell migration ability was expressed by the cell scratch rate. **a** The healing rate of NCI-H292 cell increased with the increase of CX3CL1 concentration (0, 50, 100 ng/mL). **b** The healing rate of PG cell increases with the increase of CX3CL1 concentration (0, 50, 100 ng/mL). **c** The healing rate of PGCl3 cell increased with the increase of CX3CL1 concentration (0, 50, 100 ng/mL). Compared with the control group, $*P < 0.05$ means statistically significant difference. The experiment was repeated three times

cases with primary lung cancer and bone metastasis in lung cancer. After detection, it was found that the expression of CX3CL1 and CX3CR1mRNA and protein in primary lung cancer and bone metastasis of lung cancer were significantly higher than those in adjacent tissues. The expression in lung cancer bone metastasis was significantly higher than that in the original lung cancer tissues, and the differences were statistically significant (both were $P < 0.0001$). Spearman correlation analysis showed that the expression of CX3CL1 and CX3CR1 proteins in lung cancer tissues is positively correlated, suggesting that the expression of the CX3CL1–CX3CR1 axis may be involved in promoting lung cancer growth and bone metastasis.

In this study, the levels of serum CX3CL1 and CX3CR1 were measured in 100 patients. It was found that among 100 patients with lung cancer, 55 patients with lung adenocarcinoma, and 45 patients with lung squamous cell carcinoma, the expression levels of CX3CL1 and CX3CR1 in the serum of patients with bone metastasis were higher than those in patients without bone metastasis ($P < 0.05$). There was no statistically significant difference in the levels of serum CX3CL1 and CX3CR1 among patients with lung cancer, lung adenocarcinoma, and lung squamous cell carcinoma with different ages, genders, pathological types, and primary tumor sizes ($P < 0.05$), suggesting that the CX3CL1–CX3CR1 axis may be involved in the process of promoting bone metastasis in lung cancer.

This study found that the levels of serum CX3CL1 and CX3CR1 in the bone metastasis group were higher than those in the non-bone metastasis group and the healthy control group. As the level of bone metastasis increased, the levels of serum CX3CL1 and CX3CR1 also increased, suggesting that there was a positive correlation between the serum CX3CL1–CX3CR1 axis levels and the progression of bone metastasis in lung cancer ($P < 0.01$).

To further verify the results of clinical research, cell experiments were performed in this study. Cell scratch test to human lung cancer high metastatic cell line 95-D cells were treated with different concentrations (0, 50, 100, 200, 300 ng/mL) of CX3CL1 recombinant protein for 24 h. The results showed that the migration ability of the cells gradually increased with increasing concentration. Western Blot analysis detected changes in the expression level of the receptor CX3CR1 in 95-D cells with the increase of CX3CL1 recombinant protein concentration. Cell scratch experiment detected changes in the migration of CX3CL1 on 95-D cells after CX3CR1 expression was down-regulated. The results showed that the expression of CX3CR1 in 95/D cells increased with the increase of CX3CL1 concentration after treatment with different concentrations of CX3CL1 recombinant protein for 24 h. After sh-CX3CR1 transfer down-regulated the expression of CX3CR1 in 95-D cells, the effect of CX3CL1 on the mobility of 95-D cells decreased. There are limitations in this study. Only one type of human lung cancer high-metastasis cell line was selected for research. In future study, a variety of cell lines will be selected for joint verification, especially human lung cancer high-metastasis cell lines with bone metastasis.

In summary, the detection of the levels of serum CX3CL1 and CX3CR1 in patients with lung cancer can improve the diagnostic efficiency of bone metastasis in lung cancer. Therefore, in the early diagnosis of lung cancer bone metastasis, the detection of serum markers CX3CL1 and CX3CR1 can be an important auxiliary for bone scans in patients with lung cancer detection tools. In this study, only two serum markers of CX3CL1 and CX3CR1 were detected, and a prospective study was needed to evaluate the feasibility of the two molecules for early diagnosis of bone metastasis of lung cancer. At the same time, the next step is to select the serum marker combined with some molecular markers of bone formation or absorption to study, which is helpful to the early diagnosis of bone metastasis of lung cancer.

Author contribution YL, HM and TD are responsible for the conception or design of the work. YL, YY, LS and WW contribute the acquisition, analysis, or interpretation of data for the work. HM and YY provide the tissue samples. TD helps in the follow-up of the patients. LS helps in reviewing the histopathology slides. All authors finally approved the manuscript version to be published. YL is the guarantor of the article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval (Research involving human participants and/or animals) The study was approved by Ethical Committee of The First Affiliated Hospital of Harbin Medical University and conducted in accordance with the ethical standards.

Informed consent All study subjects signed informed consent prior to their participation.

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