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Original Article

Expression and clinical significance of bFGF and CCL18 in non-small cell lung cancer

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Abstract: Both basic fibroblast growth factor (bFGF) and CC chemokine ligand 18 (CCL18) are important extracellular molecules in tumor microenvironment. However, their involvement in non-small cell lung cancer (NSCLC) has not been fully elucidated. The aim of the present study was to investigate the expression clinical significance of bFGF and CCL18 in NSCLC. Expression of bFGF and CCL18 protein was detected by immunohistochemistry staining. Expression of bFGF protein was significantly higher in NSCLC tissues (73.75%) than that in adjacent benign lung tissues (15.00%). bFGF upregulation was correlated with differentiation, tumor stage and lymph node metastasis ($P < 0.01$). The expression of bFGF was not related to gender, age, smoking, tumor size and histological subtype. Expression of CCL18 was significantly elevated in NSCLC tissues (66.25%) compared with that in adjacent benign lung tissues (10.00%). High expression of CCL18 was correlated with tumor stage and lymph node metastasis ($P < 0.01$). The expression of CCL18 was not related to gender, age, smoking, tumor size, histological subtype and differentiation. There was positive correlation between the expression of bFGF and CCL18 in NSCLC ($r = 0.364$, $P < 0.01$). Combination of bFGF with CCL18 might provide a new evaluation molecular marker and offer possibility to individualize treatment regimens in NSCLC.

Keyword: Basic fibroblast growth factor (bFGF), CC chemokine ligand 18 (CCL18), non-small cell lung cancer (NSCLC), immunohistochemistry

Introduction

Lung cancer is the first most common cancer and the leading cancer-related cause of mortality worldwide, with about 85% of the patients diagnosed as non-small cell lung cancer (NSCLC) [1]. Since aggressive biological characteristics and lacking of effective screening programs of NSCLC, more than two-thirds of patients are initially diagnosed as advanced stage. The median survival time of advanced NSCLC was only 8-10 months [2]. Therefore, investigating the tumor biology and the tumor microenvironment that could potentially find novel therapeutic targets and change the treatment paradigm of NSCLC.

Tumor angiogenesis is an important biological process and a relatively early event during lung cancer pathogenesis. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), participate in tumor angiogenesis [3]. VEGF is a vascular permeability factor secreted by tumor cells. VEGF activates mainly two tyrosine kinase receptors: VEGFR-1 and VEGFR-2. VEGFR-1, expressed in vasculature, can act as a negative regulator of angiogenesis, while VEGFR-2 plays a primary role in angiogenesis [4]. bFGF is another well known inducer of angiogenesis, with a complex biological effect acting through transmembrane tyrosine kinase receptors (mainly fibroblast growth factor receptors FGFR-1 and FGFR-2) with high affinity [5]. bFGF and VEGF work synergistically in vitro and in vivo [6]. bFGF is a potent mitogen and a survival factor in some experimental models that are of potential relevance in cancer biology [7]. The FGFRs binding to fibroblast growth factor (FGF) lead auto phosphorylation of intracellular tyrosine residues,

Table 1. The variable code and assignment situation of multivariate logistic regression

Variables	Clinical characteristics	Assignment situation
Y1	Sex	0 = male 1 = Female
Y2	Age (years)	0 ≤ 60 1 ≥ 60
Y3	Smoking status	0 = Control 1 = Smoking
Y4	Tumor size	0 ≤ 3 cm 1 ≥ 3 cm
Y5	Pathological type	0 = Adenoca. 1 = Squamous ca.
Y6	Differentiation	0 = Low 1 = High/middle
Y7	Stage	0 = I + II 1 = III + IV
Y8	Lymph node metastasis	0 = No 1 = Yes
X1	bFGF	0 = - 1 = + 2 = ++ 3 = +++
X2	CCL18	0 = - 1 = + 2 = ++ 3 = +++

subsequently activating various signaling pathways downstream of FGFR, which is involved in cell migration, cell differentiation, and instigating tumor cell proliferation, invasion, and survival in various tumor types [8]. Anti-angiogenic agents are used in treating patients with NSCLC, and their molecular biomarkers are also being assessed to predict response. More studies about anti-angiogenic agents are focus on VEGF in NSCLC at present, studies about bFGF in NSCLC are not fully illustrated [9]. Chemokines, a family of chemotactic cytokines, play a role in various biological and pathological processes, such as migration of leukocytes, angiogenesis, and tumor growth [10]. As a novel defined member of CC chemokines, CC chemokine ligand 18 (CCL18) has been demonstrated to promote invasion of cancer cells by triggering integrin clustering and enhancing their adherence to the extracellular matrix (ECM) [11]. CCL18 could promote the tumor malignant progression of breast cancer [11], ovarian cancer [12], bladder cancer [13] and pancreatic cancer [14]. The expression of CCL18 upregulation could predict poor prognosis in these cancers. Thus, the goal of our study is to research the clinicopathological characteristics of bFGF and CCL18 in NSCLC.

Material and methods

Patients and tissues

The study was approved by the Research Ethics Committee of First Affiliated Hospital of Jinan University, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards. 80 samples of NSCLC paraffin-embedded organization blocks were collected from December 2008 to March 2013, which were confirmed by the department of pathology. Among them, there were 52 males and 28 females. Mean age of the patients was 63 years. Smoking status: smoking quantity more than 20 cigarettes per day and smoking time ≥ 20 years was 36 cases, the other as the control group was 44 cases. Tumor size: the diameter ≤ 3 cm was 39 cases, > 3 cm was 41 cases. Pathological types: adenocarcinoma was diagnosed in 42 patients, 38 patients were presented with squamous carcinoma. 43 patients were diagnosed with high/middle differentiation, 37 patients with low differentiation. According to the IASLC released the seventh edition of TNM staging system for NSCLC in 2009, we found 49 patients in stage I and stage II, 31 patients in stage III and stage IV. 45 patients were no lymph node metastasis, 35 patients were lymph node metastasis. All of the enrolled patients were not received any anticancer therapy. In addition, 20 cases of carcinoma adjacent tissue (away from the tumor > 5 cm), pathologically confirmed as normal lung tissue, was a normal control group.

Main reagents

Rabbit anti human bFGF polyclonal antibody (1:10) and Rabbit anti human CCL18 polyclonal antibody (1:500) were procured from the American R&D company. According to the antibody instructions, 0.5 ug/ml antibody was chosen as the suitable working concentration. The second generation immunohistochemistry kit, DAB color enhancing agent were purchased from Fujian Maixin Biotechnology Development Company. Antibody diluent, PBS buffer, xylene, ethanol, citric acid repair solution and distilled water were provided by the department of pathology in the first Affiliated Hospital of Jinan University.

Experiment methods and the standard of judgment

The slides were deparaffinized in xylene and hydrated through graded ethanol to deionized water. Endogenous peroxidase activity was

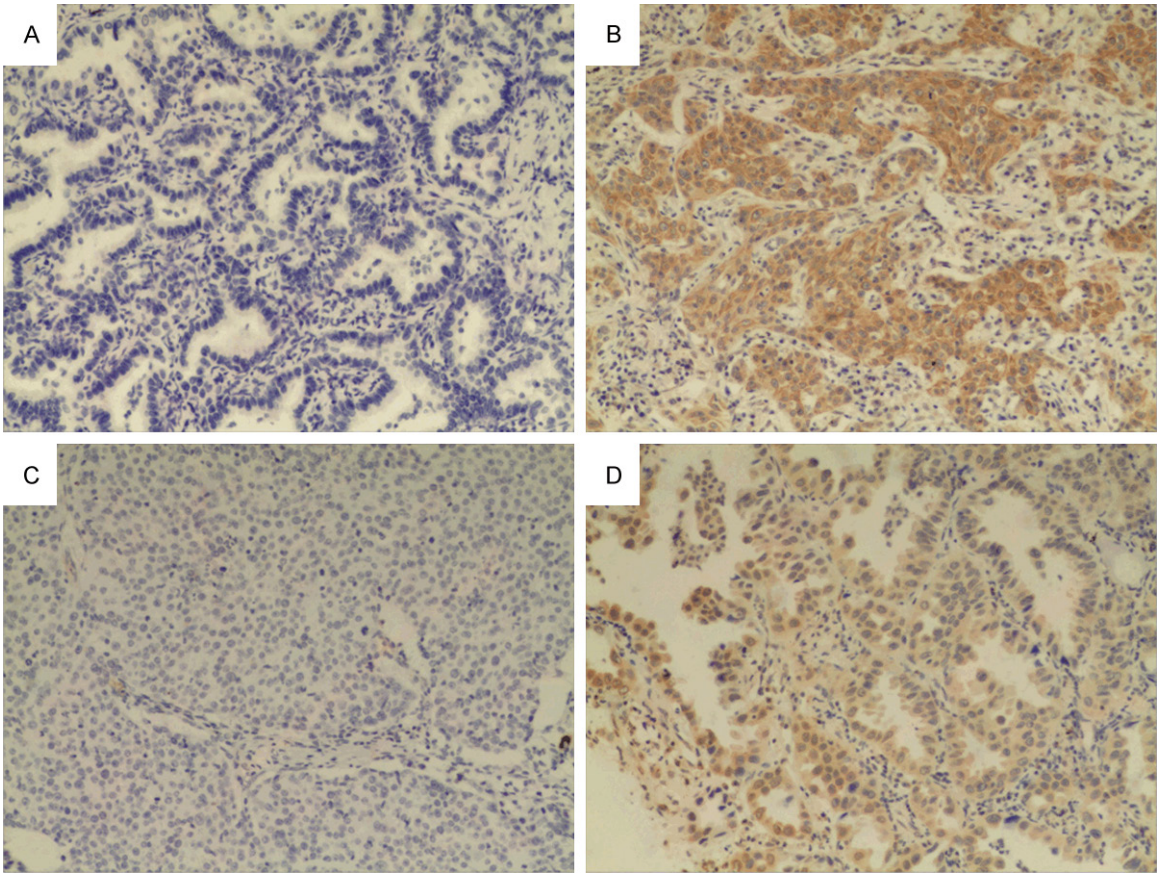

Figure 1. Immunohistochemistry staining of bFGF and CCL18 in the tissue of NSCLC. A. The negative expression of bFGF in NSCLC (×100); B. The positive expression of bFGF in NSCLC (×100); C. The negative expression of CCL18 in NSCLC (×100); D. The positive expression of CCL18 in NSCLC (×100).

Table 2. The positive expressions of bFGF and CCL18 in NSCLC and normal lung tissue

Group	N	bFGF				CCL18			
		+	Proportion (%)	x^2	P	+	Proportion (%)	x^2	P
NSCLC	80	59	73.75	23.44	0.000	53	66.25	20.46	0.000
Control	20	3	15.00			2	10.00		

blocked by 5-min incubation in 3% hydrogen peroxide-methanol buffer. Antigens were retrieved by boiling the slides in a steamer with sodium citrate buffer (pH 6.0) for 20 min. The slides were next incubated with tris buffered saline (TBS) for 20 min at room temperature to reduce nonspecific background staining. The primary antibodies were applied for 1 h at room temperature in 1× TBS. After a series of 1× TBS rinses, secondary antibody was detected by using an anti horseradish peroxidase-labeled polymer secondary antibody from the EnVision system. The slides were stained for 5-10 min with DAB Kit. Finally, the slides were counterstained with hematoxylin and eosin. Then immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological data and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through reexamining the staining by both pathologists to achieve a consensus score. The number of positive-staining cells in ten representative microscopic fields was counted and the percentage of positive cells was calculated. Given the homogenicity of the staining of the target proteins, tumor specimens were scored in a semiquantitative manner. Integral cut sheets were observed by 10 high power fields randomly. Positive cells ≥ 10% was defined as positive [15].

Statistical analysis

SPSS 13.0 software was applied in our study to perform the statistical analysis. The relativity between the expression of bFGF and CCL18 in NSCLC and clinical pathological parameters of patients were performed with chi-square test. The Spearman rank correlation analysis was used to analyze the relativity between the expression of bFGF and CCL18 in NSCLC. Multivariate logistic regression was performed to identify role of bFGF and CCL18 in NSCLC. The variable code and assignment situation of multivariate logistic regression were summarized in **Table 1**. $P < 0.05$ was consider as statistically significant.

Results

The expressions of bFGF and CCL18 in NSCLC and normal lung tissue

Immunohistochemistry staining was performed to detect the protein expression of bFGF and CCL18 in 80 NSCLC tissues and 20 adjacent benign lung tissues. Our study showed that bFGF localized in the cytoplasm. Brown and yellow granules appeared in the cytoplasm of the positive cells, occasionally visible in nucleus, (**Figure 1**). The positive expression rate of bFGF in NSCLC tissues was 73.75%, which was significantly higher than that (15.00%) in adjacent benign lung tissues, and the difference between the two groups was statistically significant ($x^2 = 23.44$, $P = 0.000$), (**Table 2**). Our study results showed that CCL18 localized in the cytoplasm, brown and yellow granules appear in the cytoplasm of the positive cells, (**Figure 1**). The positive expression rate of CCL18 in NSCLC tissues was 66.25%, which was significantly higher than that (10.00%) in adjacent benign lung tissues, and the difference between the two groups was statistically significant ($x^2 = 20.46$, $P = 0.000$), (**Table 2**).

Relationship between the bFGF and CCL18 expressions and the clinical characteristics of NSCLC patient

Expression of bFGF in NSCLC was correlated with differentiation ($x^2 = 8.476$, $P = 0.004$), clinical stage ($x^2 = 7.180$, $P = 0.007$) and lymph node metastasis ($x^2 = 7.060$, $P = 0.008$), but no obvious relativity were found between bFGF expression and gender, age, smoking, tumor size and histological subtype, (**Table 3**).

Expression of CCL18 in NSCLC was correlated with clinical stage ($x^2 = 7.028$, $P = 0.008$) and lymph node metastasis ($x^2 = 7.675$, $P = 0.006$), but no obvious relativity were found between CCL18 expression and gender, age, smoking, tumor size, histological subtype and differentiation, (**Table 3**).

The relations between the expression of bFGF and CCL18 in NSCLC

Our study results showed that there was positive correlation between the expression of bFGF and CCL18 in NSCLC ($r = 0.364$, $P = 0.001$), (**Table 4**).

The multivariate logistic regression of bFGF and CCL18 in NSCLC

The results in our study indicated that the expression of bFGF and CCL18 played an important role in differentiation, stage and lymph node metastasis in NSCLC ($P < 0.05$), (**Table 5**).

Discussion

Since the highly progressive and metastasized character of NSCLC, the overall five-year survival rate of NCSLC is only 15%. Metastasis represents the major cause of death from lung cancer, responsible for 90% of all morbidity [16]. Currently research on NSCLC metastasis has shifted from studying cancer-cell autonomous functions to tumor microenvironment: stromal cells, extracellular matrix (ECM) and signaling molecules. Discovery of the mechanisms of micro-environmental interactions will provide new biomarkers and potential drug targets for NSCLC treatment.

The FGF family represents a group of heparin-binding, multifunctional polypeptides, which also are commonly found in malignant tumors [17]. bFGF is an important extracellular molecule in tumor microenvironment. bFGF is considered a potent stimulator of angiogenesis and binds with high affinity mainly to its receptor FGFR-1, a tyrosine kinase receptor [18]. bFGF can induce the proliferation of tumor cells in autocrine or paracrine form. bFGF can also promote the proliferation of vascular endothelial cells and stimulate the chemotactic movement of endothelial cells to tumor tissue, thus contributing to angiogenesis which can promote tumor growth by providing nutritional sup-

Table 3. The clinical characteristics of bFGF and CCL18 expression in NSCLC patients

Clinical characteristics	N	bFGF				CCL18			
		+	Proportion (%)	x^2	P	+	Proportion (%)	x^2	P
Sex									
Male	52	39	75.00	0.120	0.729	32	61.54	1.475	0.225
Female	28	20	71.42			21	75.00		
Age (years)									
≤ 60	34	27	79.41	0.979	0.322	21	61.76	0.532	0.466
> 60	46	32	69.57			32	69.57		
Smoking status									
Smoking	36	27	75.00	0.053	0.818	24	66.67	0.005	0.943
Control	44	32	72.73			29	65.91		
Tumor size									
≤ 3 cm	39	28	71.79	0.150	0.698	28	71.79	1.046	0.306
> 3 cm	41	31	75.61			25	60.98		
Pathological type									
Adenoca.	42	28	66.67	2.292	0.130	27	64.29	0.153	0.696
Squamous ca.	38	31	81.58			26	68.42		
Differentiation									
High/middle	43	26	60.47	8.476	0.004	25	58.14	2.735	0.098
Low	37	33	89.19			28	75.68		
Stage									
I + II	49	31	63.27	7.180	0.007	27	55.10	7.028	0.008
III + IV	31	28	90.32			26	83.87		
Lymph node metastasis									
No	45	28	62.22	7.060	0.008	24	53.33	7.675	0.006
Yes	35	31	88.57			29	82.86		

Table 4. The relations between the expressions of bFGF and CCL18 in NSCLC

bFGF	CCL18				Total	r	P
	+++	++	+	-			
+++	4	8	0	2	14	0.364	0.001
++	6	6	5	7	24		
+	0	5	4	12	21		
-	0	5	10	6	21		
Total	10	24	19	27	80		

Note: r is the spearman rank correlation coefficient.

ports and eliminating metabolites [19]. The study reported high bFGF level was related to paclitaxel resistance [20]. bFGF can not only stimulate fibroblasts to produce collagen to regulate the synthesis and degradation of matrix, but also promote tumor cells to produce fibrinolysis enzyme activators to enhance the ability of tumor cells invasion to ECM [21].

CCL18 is a member of the serum-based cytokine family of secreted proteins involved in immunoregulatory and inflammatory processes, which is also an important extracellular molecule in tumor microenvironment. CCL18 is secreted by dendritic cells (DC) and monocytes/macrophages [22]. The immature dendritic cells and lymphocytes are the main target cells of CCL18 [23]. CCL18 can be preferentially expressed through DC, and can induce naive T cell (Tn) and B lymphocytes from the cortex to participate in the primary immune response [24]. In recent years, more and more studies have confirmed that CCL18 was highly expressed in some malignant tumors such as breast cancer, gastric cancer, ovarian cancer, liver cancer and leukemia. CCL18 might participate in the process of tumor proliferation, invasion, metastasis and angiogenesis. CCL18 elevated breast cancer cells to adherence on ECM, via integrin α5β1 aggregation, thereby promoting the invasion and migration of breast cancer [25].

Our study results showed that the protein levels of bFGF and CCL18 in NSCLC tissues were sig-

Table 5. The multivariate logistic regression results of bFGF and CCL18 in NSCLC

Clinical characteristics	bFGF					CCL18				
	B	SE	OR	95% CI	P	B	SE	OR	95% CI	P
Sex	0.810	0.610	2.247	0.679, 7.431	0.185	-0.566	0.608	0.568	0.172, 1.871	0.352
Age (years)	-0.034	0.602	0.966	0.297, 3.147	0.955	-0.004	0.608	0.996	0.303, 3.277	0.995
Smoking status	0.121	0.601	1.129	0.347, 3.669	0.840	-0.084	0.607	0.919	0.280, 3.019	0.889
Tumor size	0.194	0.601	1.214	0.374, 3.943	0.747	-0.271	0.607	0.762	0.232, 2.504	0.655
Pathological type	0.083	0.596	1.086	0.337, 3.495	0.890	-0.173	0.602	0.841	0.258, 2.738	0.774
Differentiation	1.431	0.601	6.963	1.338, 40.389	0.022	-2.769	0.603	8.142	2.103, 95.763	0.007
Stage	1.630	0.595	7.594	1.513, 50.703	0.012	-1.597	0.605	7.137	1.484, 47.692	0.014
Lymph node metastasis	2.953	0.607	8.431	2.346, 99.530	0.006	-1.302	0.592	6.735	1.184, 35.893	0.041

Note: B is the regression coefficient; SE is standard error; OR is odds ratio; 95% CI is 95% confidence interval.

nificantly higher than those in noncancerous lung tissues. We observed that bFGF upregulation was associated with differentiation, clinical stage and lymph node metastasis. CCL18 expression was related to clinical stage and lymph node metastasis. The expression of bFGF was related to CCL18 in NSCLC. Our study revealed bFGF and CCL18 may participate in the infiltration, invasion and metastasis of NSCLC. Studies have shown that, bFGF could regulate the role of RNA polymerase I, thereby promoting the division and proliferation of tumor cell [26]; and CCL18 could also enhance the proliferation of tumor cells, and to a certain extent, promote heteroploid changes of cells [27]. bFGF could promote the proliferation of fibroblasts, and could stimulate and regulate the proliferation and differentiation of fibroblasts [26]. While CCL18 could produce a chemotactic effect on fibroblasts [28], and activate the biological reactions of fibroblasts [29]. In terms of improving the infiltration and invasion of tumors, bFGF was the first identified angiogenesis factor, playing a strong regulatory role in the angiogenesis of tumor [30], and bFGF could promote tumor cells to produce fibrinolysis enzyme activators to enhance the ability of tumor cells invasion to ECM [18]. While a study of chorioallantoic membrane (CAM) angiogenesis indicated that mononuclear cells induced by CCL18 could significantly promote the formation of tumor neovascularization [31], and CCL18 elevated breast cancer cells to adherence on ECM, via integrin α5β1 aggregation, thereby promoting the invasion and migration of breast cancer [25]. In addition, one of the major signal transduction pathways of bFGF/FGFR-1 is PI3K pathway. Researches showed that CCL18 could bind to and potently activate G-protein-coupled receptors, and activate PI3K signal transduction pathway [32]. Thus, PI3K pathway may be the common signal transduction pathway of bFGF and CCL18. bFGF and CCL18 may have a certain correlation in biological activity, signal transduction and promote tumor progress, invasion and migration together.

Conclusion

Our study investigated the protein levels of bFGF and CCL18 in patients with NSCLC. There was an increase of expression of bFGF and CCL18, which were correlated with clinical stage and lymph node metastasis. There was positive correlation between the expression of bFGF and CCL18 in NSCLC. Our data showed the convincing evidence that the upregulation of bFGF and CCL18 may be involved in the tumor aggressive progression of NSCLC. bFGF and CCL18 may interact together to promote infiltration, invasion and metastasis in NSCLC. bFGF and CCL18 are important extracellular molecule in tumor microenvironment of lung cancer and might be potential targets in the therapy of lung cancer.

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Disclosure of conflict of interest

None.

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