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ORIGINAL ARTICLE

Abnormal expression of VEGF and its gene transcription status as diagnostic indicators in patients with non-small cell lung cancer*

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Abstract

Objective Angiogenesis is known to be essential for the survival, growth, invasion, and metastasis of lung cancer cells. Vascular endothelial growth factor (VEGF) is an important factor regulating angiogenesis of non-small cell lung cancer (NSCLC); however, its pathologic features and significance are unclear. In this study, the tissue VEGF expression levels and its gene transcriptional status, as well as circulating VEGF levels, were investigated in patients with lung disease.

Methods VEGF protein and mRNA expression levels in 38 lung tissue samples were analyzed by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR), respectively. Circulating VEGF levels were detected quantitatively by an enzyme linked immuno-sorbent assay.

Results The level of VEGF expression was significantly higher in lung cancer tissue than in the corresponding paracancerous or non-cancerous tissues. The average level of VEGF-positive staining was 76% in tissue samples from NSCLC patients; the levels were 89% in tissue samples from stage III patients and 92% in stage IV patients. High VEGF expression was also evident in cases with lymph node metastasis (84%), distant metastasis (90%), and lower differentiation degree (89%). VEGF mRNA in cancerous tissues was represented predominantly by the $VEGF_{121}$ and $VEGF_{165}$ isoforms. Circulating VEGF levels were significantly higher in NSCLC patients [(840 ± 324) pg/mL] than in patients with benign lung diseases [(308 ± 96) pg/mL] or in healthy individuals serving as controls [(252 ± 108) pg/mL].

Conclusion The over-expression of lung VEGF and its gene transcription status should be useful molecular indicators for NSCLC diagnosis.

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Key words: lung cancer; vascular endothelial growth factor (VEGF); VEGF mRNA; reverse transcription-polymerase chain reaction (RT-PCR); immunohistochemistry

Angiogenesis, the formation of new blood vessels from the existing vascular bed, is an essential step in the growth and invasion of the primary tumor [1–2]. Vascular endothelial growth factor (VEGF) belongs to a family of cytokines (VEGF-A, B, C, and D), signaling through diverse receptors (VEGF-R1, R2, and R3), such that it has multiple functions [3–4]. VEGF stimulates the proliferation of endothelial cells, thereby playing an important role in tumor angiogenesis and metastasis and influencing patient prognosis. Lung cancer is one of the most common malignant tumors in China. Increased serum VEGF expression levels may be associated with the clinical stage and prognosis in patients with non-small cell lung cancer (NSCLC) [5–6]. On one hand, VEGF can specifically induce endothelial cells and directly stimulate angiogenesis [7–8]; on the other hand, it can induce an increase in the permeability of blood vessels causing macromolecule to leak out of the vessels and resulting in the formation of matrixes outside the blood vessel, which provides a frame for proliferation and angiogenesis, thereby playing an indi-

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rect role in angiogenesis [9]. In addition, VEGF can attract macrophages by chemotaxis and stimulate these cells to release angiogenesis factors, as a further means of indirectly stimulating angiogenesis [10–11].

The role of VEGF during the development and progression of lung cancer has been strictly related to angiogenesis and the protein has been implicated in tumorigenesis and metastasis due to its known role in mediating the proliferation of endothelial cells and promoting vascular permeability, making it an important regulator of lung carcinomas [12–13]. However, the diagnostic and distinguishing value of VEGF overexpression and its gene transcriptional status in patients with lung cancer remains controversial. In the present study, VEGF expression and cellular distribution, its gene transcription status in different tissues, as well as circulating VEGF levels in patients with lung disease, were investigated in order to analyze how the cytokine influences the pathological and clinical characteristics of lung cancer.

Materials and methods

Lung tissue and serum samples

For this study, fresh lung cancerous tissue (T), self-controlled adjacent cancerous tissue (A), and non-cancerous tissue (N) were obtained from 38 patients who underwent surgery for lung cancer at the Affiliated Hospital, Nantong University, China from July 2013 to July 2014. All lung specimens were collected from patients (32 men and 6 women, ranging in age from 40 to 68 years) diagnosed with NSCLC according to histological classification, including 24 cases of squamous carcinoma, 10 cases of adenocarcinoma, 3 cases of squamous adenocarcinoma, and 1 case of bronchioloalveolar carcinoma. According to TNM staging [14], the patient cohort included 10 patients with stage-I, 6 with stage-II, 9 with stage-III, and 13 with stage-IV NSCLC. The tissue specimens were immediately frozen in liquid nitrogen and kept at –85 ℃ until required. Each of the latter specimens was divided into two parts; one half was used for immunohistochemical staining and the other was fixed in formalin, embedded in paraffin, and then cut into 4–5-μm-thick sections for pathological examination (hematoxylin and eosin staining).

Serum samples and full clinical records were obtained from the 38 patients with lung cancer. Preoperative peripheral venous blood was collected from these patients, and the separated sera were stored at –85 ℃ until required. Serum VEGF levels were simultaneously analyzed for the 38 lung cancer patients as well as for 30 patients with benign lung diseases (mainly inflammatory disease: 20 men and 10 women, ranging in age from 39 to 65 years) registered at the Hospital, and 30 healthy volunteers (normal controls: 20 men and 10 women, ranging in age from 36 to 60 years) from the Central Blood Bank of Nantong City, China.

Immunohistochemical analysis

The UltraSensitive™ streptavidin peroxidase (S-P) kit and positive control were purchased from Maixin Biotechnology Development Company (Fuzhou, China). Five-micron paraffin-embedded tissue sections were deparaffinized in xylene and dehydrated using a serial gradient of ethanol solutions. Thereafter, S-P immunohistochemical analysis was performed according to the manufacturer's instructions. Finally, tissue sections were restained with hematoxylin and observed. The negative controls included either normal rabbit IgG in place of the primary antibody or secondary antibody alone. VEGF staining of tissue sections was evaluated semi-quantitatively according to the percentage of the total cells that were VEGF-positive, and classified as follows: diffusely positive (> 50% VEGF-positive cells), moderately positive (16%–50% VEGF-positive cells), weakly positive (5%–15% VEGF-positive cells), and negative (< 5% VEGF-positive cells).

Extraction of tissue total RNA and synthesis of cDNA

The resected tissues (50 mg) were homogenized completely in 1 mL TRIzol™; 0.2 mL chloroform was added and the solution shaken vigorously for 15–30 s. The sample was stored on ice for 5 min and centrifuged at 12 000 g for 15 min. The supernatant was transferred to a fresh 1.5-mL Eppendorf tube and the RNA precipitated by the addition of 0.5 mL isopropanol. Precipitation of RNA occurred after 5 min on ice; following which centrifugation was carried out at 12 000 g for 5 min at 4 ℃. Thereafter, the supernatant was removed and the pellet washed with 1 mL of 750 mL/L ethanol, before centrifugation at 7500 g for 5 min at 4 ℃. Finally, after careful removal of the residual ethanol, the purified RNA was dissolved in 100 μL DEPC-H_2O and stored at –80 ℃ for future analysis. Total RNA concentrations (μg/mg of wet tissue of the lungs) were assessed by measurement of absorbance at 260 nm using an ultraviolet spectrophotometer (Shimadzu UV-2201 type, Japan).

For synthesis of cDNA, 2 μg of total purified RNA was denatured in the presence of random hexamers (200 pMol, Promega, Madison, WI) at 95 ℃ for 5 min and incubated with moloney murine leukemia virus reverse-transcriptase (GIBCO, BRL) at 23 ℃ for 10 min, 37 ℃ for 60 min and 95 ℃ for 10 min in a 20 μL reaction volume using a first-strand cDNA synthesis kit (Fermentas Co., Thermo, USA), followed by incubation on ice for 5 min and storage at –20 ℃ for future PCR analysis.

Amplification of VEGF-cDNA

The resulting cDNA was amplified by PCR using oligonucleotides designed according to the human VEGF sequence [15] and synthesized using Synthesizer (Model 381 A, Applied Biosystems, Foster City, CA). The sequences of these primers were as follows: VEGF-P1 (sense), 5'-CGAAGTGGTGAAGTTCATGGATG-3', and VEGF-P2 (antisense), 5'-TTCTGTATCAGTCTTTCCTGGTGAG-3'. The reaction was performed in a standard 50 μL reaction mixture consisting of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ (pH 8.3), 0.2 mM dNTPs, 50 pM of each sense and antisense primer and 2.5 U of Amplitag DNA polymerase. The initial denaturation step was carried out at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 30 s, primer annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 90 s. Finally, an additional extension step was carried out for 10 min at 72 ℃. As a negative control, a sample in which the DNA template was omitted was included in the PCR analysis. Expression of the housekeeping gene, *GAPDH* was used as a control for relative quantification and specific oligonucleotides were designed according to the *GAPDH* sequence in the human genome [16]. Primer sequences for *GAPDH* were as follows: GAPDH-1 (sense), 5'-ACCACAGTCCATGCCATCAC-3' (nt 601-620) and GAPDH-2 (anti-sense), 5'-TCCACCACCCTGTTGCTGTA-3' (nt 1033-1052). The anticipated size of a PCR product generated using the latter primers was 452 bp.

PCR products were electrophoresed on 1.5% agarose gels with ethidium bromide staining, and sizes were estimated using a DNA ladder (Promega). Electrophoresis images were scanned and the intensity of each band on the agarose gel was quantified using Dolphin-1D Gel Analysis Software (Wealtec Corp., USA). Expression data were compared between patient samples after being normalized according to the intensity of the band for the *GAPDH* PCR product.

Detection of serum VEGF level

The levels of VEGF in the sera of 38 patients with lung cancer, as well as those from 30 patients with benign lung diseases and 30 healthy patients (controls), were detected using an enzyme linked immuno-sorbent assay, according to the manufacturer's instructions. The system used a solid phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against recombinant human VEGF. In this assay, protein standards, controls, and serum samples were incubated in microtitre wells, which had been coated with an anti-VEGF antibody. After incubation and washing, the samples were incubated with another anti-VEGF antibody, this time a detection antibody, which is labeled with the enzyme, horseradish peroxidase. After a second incubation and washing step, the samples were incubated with the substrate, tetramethylbenzidine. An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured was directly proportional to the concentration of VEGF present in a particular sample. A set of VEGF standards was used to plot a standard curve of absorbance versus VEGF concentration, from which the VEGF concentrations in the serum samples were calculated.

Statistical analysis

Lung tissues were divided into cancerous, paracancerous and non-cancerous groups. Serum samples were divided into three groups: lung cancer, benign lung disease, and normal controls. Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by using the F test and χ^2 test. P-values < 0.05 were considered statistically significant.

Results

Pathological features of tissue VEGF expression

The brown particles representative of VEGF expression in lung cancer tissues were located in the cytoplasm and cell membranes according to immunohistochemical detection, as shown in Fig. 1. The positive VEGF expression was closely associated with lymph node metastasis, distant metastasis, and tumor differentiation degree (Table 1). The incidences of VEGF expression in different lung tissues were 76% (29/38) in the cancerous group, 21%

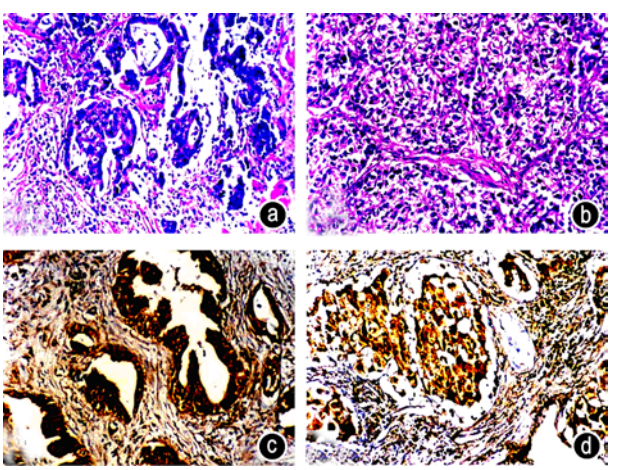

Fig. 1 Immunohistochemical analysis of VEGF expression in lung cancer tissues. (a) Lung adenocarcinoma stained by hematoxylin and eosin (H&E, 20×); (b) Lung squamous carcinoma stained by H&E (20×); (c) VEGF staining in lung adenocarcinoma represented by brown particles in the cytoplasm and cell membrane according to streptavidin peroxidase activity (SP, 200×); (d) VEGF staining in lung squamous carcinoma as brown particles in the cytoplasm and cell membrane (200×)

Table 1 Clinicopathological features of VEGF expression in lung cancer tissues (*n*)

Groups	*n*	VEGF		*P*
		Negative	Positive (%)	
Lung cancer tissues				
Cancerous	38	9	29 (76)	
Paracancerous	38	30	8 (21)	
Distal cancerous	38	34	4 (11)	< 0.01
pTNM staging				
I	10	5	5 (50)	
II	6	2	4 (67)	
III	9	1	8 (89)	
IV	13	1	12 (92)	< 0.01
Lymphatic metastasis				
Without	13	5	8 (62)	
With	25	4	21 (84)	< 0.05
Distant metastasis				
Without	28	8	20 (71)	
With	10	1	9 (90)	< 0.05
Differentiated degree				
Well	6	3	3 (50)	
Middle	13	4	9 (69)	
Poor	19	2	17 (89)	< 0.05

(8/38) in the corresponding paracancerous group, and 11% (4/38) in the corresponding non-cancerous group. The incidence of VEGF expression in the cancerous group was significantly higher ($P < 0.01$) than that in the corresponding paracancerous or distant cancerous group. In addition, there was a close relationship between tumor TNM stage and VEGF expression. The level of VEGF expression was 50% for stage I, 67% for stage II, 89% for stage III, and 92% for stage IV lung cancer samples. The level of VEGF expression was 62% (8/13) in samples from patients without lymph node metastasis, and 84% (21/25) in those with lymph node metastasis; expression was 71% (20/28) in patients without distant metastasis, and 90% (9/10) in those with distant metastasis. The higher VEGF expression was found in the patient samples that showed poor differentiation, compared to that of the middle- or well-differentiation groups of lung cancer patients.

Alterations in total RNA and VEGF-RNA in different lung tissues

The total RNA concentration of matched lung cancerous, paracancerous and distant cancerous tissues, as well as the levels of specific VEGF-RNA isoforms in these tissues, were shown in Fig. 2. A stepwise decrease in total RNA (Fig. 2a) and VEGF-mRNA (Fig. 2b) expression was found from lung cancer to paracancerous to non-cancerous tissues; with lung cancerous tissues displaying significantly higher ($P < 0.01$,) total RNA and VEGF-mRNA levels than either the corresponding paracancerous or non- cancerous tissues. Three different VEGF-RNA isoforms ($VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$) were amplified in lung tissues by RT-PCR. Analysis of *VEGF* transcription in the lung revealed the presence of VEGF-RNA in 76.3% (29/38) of the samples and products corresponding to the $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ but not $VEGF_{206}$ isoforms were found in different areas of the lung cancerous tissues. The incidences of lung VEGF isoforms were 3.4% considering $VEGF_{189}$, 17.2% considering $VEGF_{165}$, and 10.3% considering $VEGF_{121}$. In 20 out of 29 cases, the two secretory isoforms ($VEGF_{121}$ and $VEGF_{165}$) were concomitantly detected. According to the ratio of tissue *VEGF* to the housekeeping gene, *GAPDH*, the intensity of each specific band on the agarose gel was semi-quantitatively evaluated using a gel scan analyzer. The lung tissue VEGF was ladderlike expression in different parts of lung cancer tissues (Fig. 2c). The level of tissue VEGF-RNA was significantly higher in lung tumor specimens (T) than that in their adjacent tissue (A; $P < 0.05$) or in the corresponding non-cancerous tissues (N; $P < 0.01$).

Circulating VEGF levels in patients with lung diseases

The levels of serum VEGF and its clinical significance were investigated in patients with malignant or benign lung disease. The levels of circulating VEGF in patients with different types of lung disease were shown in Fig. 3. The levels of circulating VEGF were (840 ± 324) pg/mL in lung cancer patients, (308 ± 96) pg/mL in patients with benign lung diseases, and (252 ± 108) pg/mL in healthy patients used as normal controls. The serum VEGF concentration was significantly higher ($P < 0.01$) in the lung cancer group than in both the benign disease group and the control group. The expression of serum VEGF was high in patients with benign diseases before drug therapy; however, these levels significantly decreased to the normal reference range after patients received anti-inflammation medication.

Discussion

Angiogenesis is a fundamental process involved in normal organ physiology, development and tissue repair, as well as in a variety of pathological processes. When blood vessels grow, angiogenesis becomes pathologic and sustains the progression of many neoplastic and non-neoplastic diseases [14]. VEGF is a potent mitogen specific for vascular endothelial cells and may directly stimulate the growth of new blood vessels by inducing the proliferation and migration of endothelial cells under physiologic and pathologic conditions [15]. As a result, VEGF has been shown to play an important role in the angiogenesis process of various kinds of human neoplasms [16]. Angiogenesis is required for tumor growth and metastasis. Since VEGF has been reported to play an important role in this

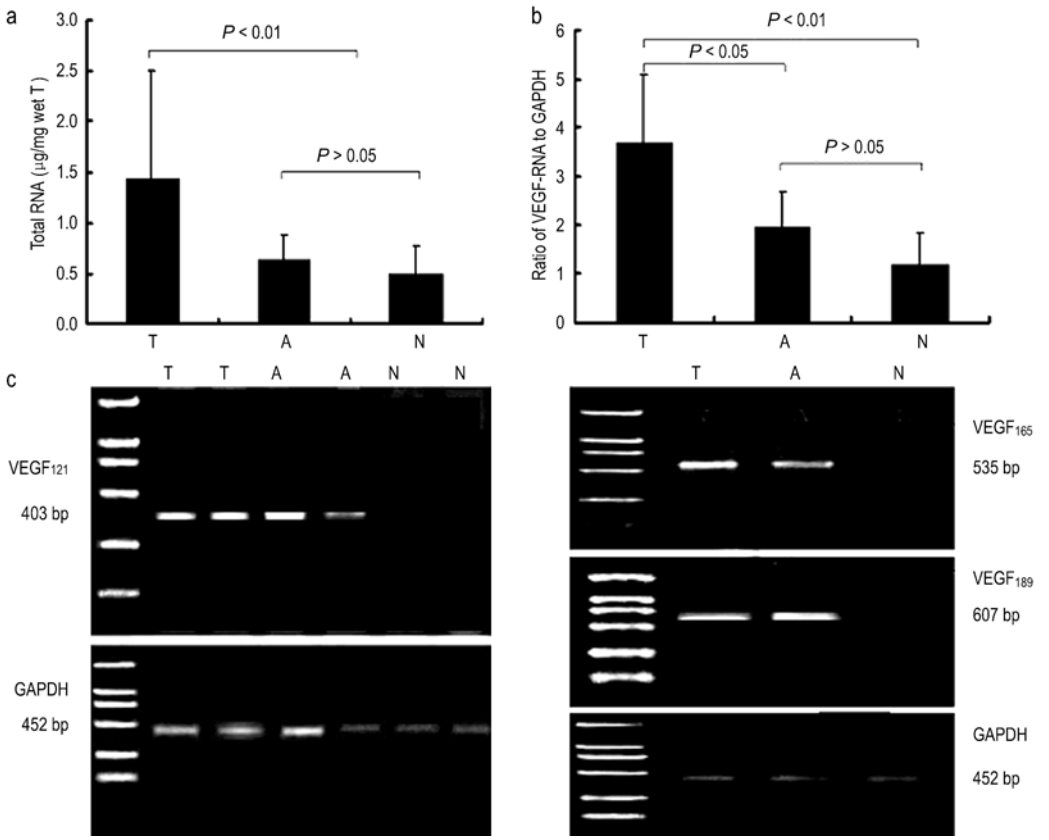

Fig. 2 Alterations in total RNA levels and VEGF-RNA expression patterns in representative lung tissues assessed by RT-PCR. (a) Total RNA levels in different lung tissues; (b) the levels of the VEGF-RNA transcript in different lung tissues; (c) Left upper: PCR amplified fragment corresponding to the $VEGF_{121}$ isoform (403 bp PCR product); Left lower: GAPDH expression used as a control (452 bp PCR product) for relative quantification of expression levels; Right upper: amplified fragment corresponding to the $VEGF_{165}$ isoform (535 bp); Right middle: amplified fragment corresponding to the $VEGF_{189}$ isoform (607 bp); Right lower: GAPDH expression (452 bp PCR product). T, cancerous tissue; A, adjacent cancerous tissue; and N, non-cancerous tissue

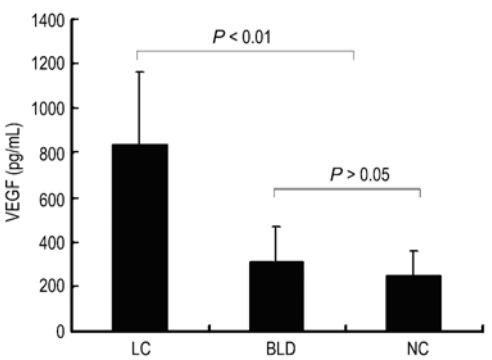

Fig. 3 Level of circulating VEGF in patients with different types of lung disease. LC, lung cancer; BLD, benign lung diseases; and NC, normal controls

angiogenesis, it is used as an independent prognostic factor for lung cancer [17]. In this study, we investigated the expression of VEGF and VEGF-RNA in different cancer tissues, as well as the VEGF level in the sera of patients with lung cancer or benign lung disease, in order to elucidate the relationship between VEGF and the pathological characteristics of lung cancer.

The relationship between VEGF expression and clinicopathological features was investigated by immunohistochemical staining. The brown particles representing VEGF expression presented in the cytoplasm and cell membranes of lung cancer cells and combination to small endothelial cells. The incidence of VEGF expression in cancerous tissues was significantly higher ($P < 0.01$) than that in the corresponding paracancerous or distant cancerous tissues. In addition, a direct relationship was found between VEGF expression and tumor pTNM stage, lymph node metastasis and differentiation degree. Indeed, significantly higher expression of VEGF was found in tissue samples from patients with lymph node metastasis than those in patients without lymph node metastasis [18–19]. The abnormal expression of VEGF was evident in tissue specimens from patients in the lower differentiation grouping and indicated that the expression of VEGF is

related not only to tumor angiogenesis but also to the malignancy of the tumor. VEGF plays an important role in tumor angiogenesis, and it has been confirmed that VEGF is an important angiogenesis factors [20].

In this study, we found that VEGF expression was significantly higher in small endothelial cells near lung cancer cells than in the corresponding paracancerous cells. Moreover, we found that high VEGF expression also correlated significantly with tumor progression and this implies that the expression of VEGF in lung cancer cells is related to prognosis in NSCLC patients. A tendency of ladder distribution from lung cancer, paracancerous to distant lung tissues was found for both VEGF protein expression and total RNA levels. We report that both total RNA and VEGF concentrations were significantly higher in cancerous tissues than in the corresponding paracancerous or non-cancerous tissues. The increase in total RNA concentration suggests an energetic protein synthesis in tumor cells and correlates with high expression of VEGF. This further suggests that VEGF is an important factor for tumor growth [21].

Human VEGF has recently been identified as a secreted endothelial cell-specific mitogen able to stimulate angiogenesis *in vivo*. Four different isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) have been described in tumors as alternative splice variants of a single gene [22]. Transcripts encoding the three shortened forms 121,165 and 189 have been detected in the majority of tumor cells expressing the VEGF gene. According to the different availabilities and affinities for their receptors, the isoforms could play different roles in the development of tumor angiogenesis. The data from this study revealed a similar pattern of VEGF isoform expression; three different cDNAs were amplified, corresponding to $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ and no band corresponding to $VEGF_{206}$ was detected. Significant association was noticed between the $VEGF_{121}$ and $VEGF_{165}$ isoforms and VEGF RNA positive rate, which are efficiently secreted and mostly stimulate the mitogenic properties of endothelial cells. Moreover, the VEGF RNA was detected in adjacent non-neoplastic tissues. This agrees with the putative biological role of VEGF in that it maintains the permeability of normal lung tissues [23]. VEGF RNA isoforms correlate with tumor angiogenesis, survival, and relapse and will be helpful in predicting the prognosis of lung cancer patients.

Tumor cells expressing VEGF may display a growth advantage and proliferate more rapidly than cells that do not express VEGF. Rapid cell proliferation in tumor tissues can lead to increased interstitial fluid pressure, which may result in compression closure of capillaries and consequent tissue necrosis [24]. Higher levels of serum VEGF have been associated with poor prognosis in patients with NSCLC, who displayed significantly higher serum levels than the control group. There were significant associations between serum VEGF level and clinicopathological parameters, histopathological diagnosis and TNM staging [25–26]. Serum VEGF level is associated significantly with clinical diagnosis and prognosis of patients with lung cancer.

VEGF is an important angiogenic factor involved in regulating angiogenesis of lung tumors. In this study, we report that the expression of VEGF was significantly higher in cancer tissues than in either the corresponding paracancerous or non-cancerous tissues, and that this higher VEGF protein level correlated strongly with total RNA and VEGF-RNA levels in these tissues. $VEGF_{121}$ and $VEGF_{165}$ were the predominant isoforms found in tumor specimens. VEGF-positive expression in lung cancer has a significant correlation with capsular infiltration, vascular invasion, and metastasis. The abnormal expression of VEGF in tissues or the circulatory system is a useful indicator for metastasis and prognosis of lung cancer [27–28].

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Conflicts of interest

The authors indicated no potential conflicts of interest.

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