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ORIGINAL ARTICLE

Clinicopathological features of hypoxia-inducible factor-1α and vascular endothelial growth factor expression in patients with lung cancer*

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Abstract

Objective The aim of the study was to investigate the clinicopathological characteristics of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression in patients with lung cancer.

Methods Cancerous and noncancerous tissues were collected post-operation from 115 patients with lung cancers by the self-control method. Total RNA was extracted from the lung tissues. The status of tissue HIF-1α expression and intercellular distribution was observed by immunochemistry using a tissue microarray. The expression levels of circulating HIF-1α and VEGF were detected by enzyme-linked immunosorbent assay (ELISA).

Results The expression of serum HIF-1α [(138.3 ± 28.8) μg/L] in the group of patients with lung cancer was significantly higher ($P < 0.01$) than that in the group of patients with pneumonia [(58.8 ± 14.5) μg/L] and the control group of patients [(24.1 ± 3.3) μg/L]. There was a strong positive correlation of serum HIF-1α levels ($r = 0.937$, $P < 0.01$) with serum VEGF levels. The specific concentration of total RNA [(1.52 ± 1.14) μg/mg wet lung tissues] in the cancerous tissues was significantly higher ($t = 8.494$, $P < 0.001$) than that in the noncancerous tissues [(0.58 ± 0.33) μg/mg]. The clinicopathological features of HIF-1α expression in lung cancer tissues revealed a significant relationship between positive HIF-1α expression and patient sex ($\chi^2 = 4.494$, $P = 0.034$), tumor size ($\chi^2 = 4.679$, $P = 0.031$), differentiation degree ($\chi^2 = 8.846$, $P = 0.012$), and presence of lymphatic node metastasis ($\chi^2 = 6.604$, $P = 0.037$).

Conclusion Abnormal HIF-1α expression in lung cancer is closely related with nucleic acid metabolism and angiogenesis, and it may be helpful in the diagnosis and identification of lung cancer.

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Key words: lung cancer; hypoxia-inducible factor-1α (HIF-1α); nucleic acid metabolism; enzyme-linked immunosorbent assay (ELISA); diagnosis

Lung cancer is one of the human malignancies with the highest incidence and the leading cause of cancer-related mortality worldwide [1–2]. Survival after lung cancer is still limited; recent studies have reported a 5-year survival rate of approximately 16% and 10-year survival rate of up to 90% when patients with lung cancer underwent prompt surgical treatment at stage I of the disease [3–4]. Angiogenesis is required for invasive tumor growth and metastasis, and plays an important role in the development and progression of lung cancer [5–6]. Cancer results in an increase in the overall oxygen consumption by cancer rapid growth, leading to a high expression of hypoxia-inducible factor-1α (HIF-1α), which stimulates angiogenesis and the release of related factors [7–8]. During hypoxic conditions, HIF-1α is dramatically stabilized and activated through a series of signaling processes, which

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then activate transcription of a network of genes that control several aspects of tumor biology, such as energy metabolism, angiogenesis, cellular growth, and apoptosis [9–10].

HIF-1α usually indicates the hypoxia index and has recently been used in the evaluation of many tumors [11–12]. The association between hypoxic tissue and vascular endothelial growth factor (VEGF), an angiogenesis-related factor, is worth exploring, as the over-expression of HIF-1α might indicate poor prognosis for patients with lung cancer [13–15], which is known to be associated with enhanced intra-tumoral hypoxia, increased tumor invasion, and increased metastasis frequency, and affects radiotherapy or chemotherapy [15–16]. However, the association between HIF-1α expression and its clinical significance in lung cancer remains to be systematically assessed. Therefore, to examine this association, in the present study, we investigated the levels of HIF-1α expression in lung tissue and circulating blood in patients with lung cancer.

Materials and methods

Lung tissues

Adenocarcinoma and noncancerous tissues were collected post-operation from 115 patients with lung cancer (58 men and 57 women; age, 39–77 years; median age, 58 years) using the self-control method at the Affiliated Hospital of Nantong University, Nantong, China from January 2012 to February 2015. All lung specimens were collected from patients diagnosed with lung cancer according to histological classification. Pathological examination of some sections (hematoxylin and eosin staining) was performed, and the rest tissues were kept at –80°C.

Serum samples

Serum samples were obtained simultaneously from these 115 patients with lung cancer. Patients with pneumonia (n = 30) and patients of a healthy group (n = 30) were used as the control subjects. The serum samples were centrifuged at 2000 rpm and stored at –80°C. We also assessed the hepatic and renal functions. The liver and kidney functions, blood lipid, and blood glucose levels were normal in the control group. All patients were followed-up as per the standards of diagnosis and treatment of patients with lung cancer, from January 2012 to February 2015.

Total RNA extraction

Lung tissue (50 mg) was extracted in 1.0 mL of TRIzol reagent (Molecular Research Center, USA) and homogenized on ice according to previous studies. The concentration of total RNA was measured by determining its optical density at 260 nm in an ultraviolet (UV) spectrophotometer and expressed as total RNA micrograms per milligram of wet tissue. The tissue was then stored at –80°C.

HIF-1α cDNA synthesis

First-strand cDNA of HIF-1α was generated using the RevertAid First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions.

Primer design and polymerase chain reaction (PCR) amplification

The primers for HIF-1α mRNA were designed according to the *HIF-1α* gene sequence (MIM603348) using the Premier Primer 5.0 software (Invitrogen, Shanghai, China). The HIF-1α primers used were forward, 5'-CTCATCCAAGAAGCCCTAAC-3' (nts 2452–2471) and reverse, 5'-TCATAACTGGTCAGCTGTGG-3' (nts 2781–2800), and the amplified fragments were 349-bp long. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for relative quantification. GAPDH primers used were as follows: forward, 5'-CACTGG CGTCTTCACCACCAT-3' (nts 396–416) and reverse, 5'-GTGCAGGAGGCATTGCTGAT-3' (nts 541–560), and the amplification sequence was 165-bp long. HIF-1α cDNA was amplified using nested PCR. Briefly, 1 μL cDNA (0.1 μg/μL), Each 1.0 μL HIF-1α forward and reverse primers (10 μmoL/L), 12.5 μL Premix Taq amplitag DNA polymerase (TaKaRa, Japan), and ddH_2O were added to a reaction tube to make a total volume of 20 μL. The conditions for PCR amplification were as follow: 5 min of pre-denaturation at 94 °C; 35 cycles amplification with TC-412 (Techne, England) at 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min; a final extension at 72 °C for 10 min, and storage at 4 °C. The amplified fragments of the *HIF-1α* gene were electrophoresed on a 1.5% agarose gel (PowerPac, Mini Trans-Blot, Bio-Rad, Laboratories, Inc., USA) and observed using a UV transilluminator at 320 nm (170-2525, Bio-Rad, Laboratories, Inc.).

DNA sequencing

HIF-1α DNA was purified using Montage™ PCR centrifugal filter devices (4 °C, 9000 rpm, 10 min), and DNA was prepared using a MegaBACE DNA sequencer (USA) with the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Bioscience, USA) according to the manufacturer's protocol. Subsequently, 1.0 μg tested DNA, 2.5 μL sequencing primer, 8.0 μL dNTP mixed reagent, and 20 μL ddH_2O were added to a reaction tube. The cycling conditions were as follows: 95 °C for 30 s; 30 cycles of 95 °C for 20 s, 50 °C for 15 s, and 60 °C for 1 min; and storage at 15 °C. DNA was precipitated using ammonium acetate and ethanol, sequenced using the

MegaBACE sequencing system (version 3.0, Amersham Biosciences), edited, and aligned with the original sequences of the *HIF-1α* gene from GenBank.

Tissue microarrays (TMA)

A 20 × 12 matrix of tissue microarray paraffin blocks was prepared, and 4-μm–thick sections were prepared from paraffin-embedded tissue blocks. Four points were allotted to each case of lung cancer and 2 points to each case of noncancerous tissue. Cells were adhered to glass slides that were coated with poly-lysine.

Immunohistochemistry (IHC)

Immunohistochemical staining (S-P method) was performed using Immunostain EliVision kit (Beijing Zhongshan Biotechnology Company, China) according to the manufacturer's instructions. Primary rabbit anti-human VEGF (BA0407) and HIF-1α (PB0245) polyclonal antibodies and mouse anti-rabbit secondary antibody IgG-Biotin (BM2004) were purchased from Wuhan Boster Biological Technology Co., China. The TMA slides were deparaffinized and dehydrated, and the deparaffinized lung sections were washed with ethylene diamine tetraacetric acid buffer and quenched in a microwave for 10 min. The sections were then incubated for 60 min with primary rabbit anti-human VEGF and HIF-1α polyclonal antibodies at room temperature, washed three times with phosphate-buffered saline (PBS), and incubated for 20 min with polymer reinforcing agent. Subsequently, the sections were rinsed in PBS thrice, incubated with mouse anti-rabbit secondary antibody IgG-Biotin for 30 min at room temperature, and developed with 0.1% 3,3'-diaminobenzidine for 5 min after washing with PBS thrice. Thereafter, the slides were rinsed with distilled water, counterstained, dehydrated, air dried, and mounted. For the negative control reactions, the primary and secondary antibodies was instead with PBS (pH = 7.5).

Evaluation of the IHC findings

The results of IHC staining were assessed by two methods: the intensity of staining and the percentage of positive cells (positive cell average in 5 fields of vision). Staining intensity was categorized into 4 groups: 0 (negative), 1 (pale yellow), 2 (brown), and 3 (dark brown). The percentage of positive cells was scored as follows: 0 (0–5%), 1 (6%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (> 75%). The product of the percentage and intensity score was defined as the final IHC staining score: 1–4 (low expression), 5–8 (moderate expression), and 9–12 (high expression).

Western blotting

The amount of total protein was determined using a bicinchoninic acid protein-measuring kit (Beyotime, China). Protein (50 μg) from each sample was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 20 V for 30 min and then at 120 V for 60 min. The protein was then transferred onto a polyvinylidene difluoride membrane and blocked by incubation with 5% nonfat dry milk in Tris-buffered saline (2.5 g nonfat dry milk and 50 mg of Tris-buffered saline with Tween-20) for 1 h in a glass dish while shaking at room temperature. The transferred membrane was incubated with mouse polyclonal antibody against human HIF-1α (1:500, Abcam, USA) or β-actin overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibodies (Beyotime, China) for 2 h at room temperature. Imaging was performed using Quantity One software (Bio-Rad, Laboratories, Inc., USA).

Enzyme-linked immunosorbent assay (ELISA)

The levels of VEGF and HIF-1α proteins were detected in the sera of patients with lung cancer using an ELISA assay kit KIT/96T (Uscnk, Wuhan, China) according to the manufacturer's instructions. The detection ranges were as follows: VEGF (SEA143Hu), 15.63–1000 pg/mL and HIF-1α (SEA798Hu), 0.156–10 ng/mL. According to the manufacturer's instructions, the absorbance (*A* value) was detected at 450 nm, and the concentrations of VEGF and HIF-1 were calculated on the basis of their respective standard curves.

Statistical analysis

Data were expressed as mean ± standard deviation, and all statistical analyses were carried out using Graphpad Prism 5.0 and SPSS software (version 18.0). A *t*-test was used to analyze protein expression in the two groups, and single-factor analysis of variance (one-way) was used to analyze the expression between groups. Furthermore, linear correlation analysis was used to analyze the relationship between HIF-1α expression and VEGF expression. Statistical significance was set at $P < 0.05$.

Results

HIF-1α and VEGF expression and correlation analysis

The serum levels of HIF-1α and VEGF in patients with benign and malignant lung diseases were shown in Table 1. From normal condition or pneumonia to adenocarcinoma, the expression level of HIF-1α increased gradually. The serum HIF-1α expression [(138.3 ± 28.8) μg/L] in the lung cancer group was significantly higher ($P < 0.01$) than that in the pneumonia group [(58.8 ± 14.5) μg/L] and the control group [(24.1 ± 3.3) μg/L], and the expression in the pneumonia group was significantly higher than that in the control group. The expressions of VEGF and HIF-

Table 1 Quantitative analysis of serum HIF-1 and VEGF expressions in patients with lung disease (*n*)

Groups	*n*	HIF-1α		VEGF	
		Mean ± SD (μg/L)	> 100 μg/L (%)	Mean ± SD (μg/L)	> 280 μg/L (%)
Lung cancer	115	138.3 ± 28.8	104 (90.4)	394.9 ± 107.3	114 (87.0)
Pneumonia	30	58.8 ± 14.5*	0 (0.0)*	162.3 ± 79.3*	4 (13.3)*
Control	30	24.1 ± 3.3**	0 (0.0)*	140.9 ± 54.5*	0 (0.0)**

Note: compared with the lung cancer group, * $P < 0.01$; ** $P < 0.001$

1α were higher in adenocarcinoma patients, considering 100.0 μg/L and 280.0 μg/L as detection limits, respectively; furthermore, the incidences of VEGF and HIF-1α were, respectively, 90.4% and 87.0% in the lung cancer group and 0.0% and 13.3% in the pneumonia group, whereas the corresponding control group showed normal levels of the both factors. There was a close relationship between the HIF-1α and VEGF expressions in patients with lung cancer ($r = 0.937$, $P < 0.01$).

HIF-1α gene and protein level in lung cancer tissue

The expression levels of the *HIF-1α* gene and protein in cancerous and para-cancerous tissues were shown in Fig. 1. The specific concentration of total RNA [(1.52 ± 1.14) μg/mg wet lung tissues] in the 115 cases of cancerous tissues was significantly higher ($t = 8.494$, $P < 0.001$) than that in the noncancerous tissues [(0.58 ± 0.33) μg/mg], and the difference between them was significant ($t = 8.4937$, $P < 0.001$). The amplified gene fragment was 349-bp long, and the sequence was consistent with that of HIF-1α from GenBank (Fig. 1a). The expression of HIF-1α in cancer tissue was significantly higher than that in para-carcinoma tissue (wet-weight tissue per mg, Fig. 1b).

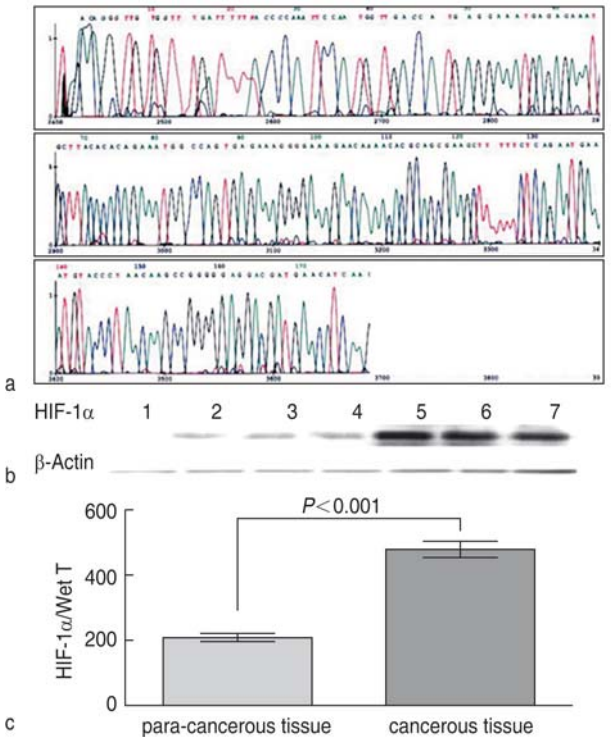

Fig. 1 Expression levels of the *HIF-1α* gene and protein in cancerous and the para-cancerous tissues. (a) Amplified *HIF-1α* gene fragment analysis; (b) Western blotting: normal control (1), para-carcinoma tissues (2–4), and cancer tissues (5–7); (c) Expression of HIF-1α in wet-weight lung tissue

HIF-1α expression in lung cancer tissues

Tissue matrix was organized in an order on the TMA and stained by IHC S-P (Fig. 2). IHC showed that brown particles, representative of HIF-1α expression, were

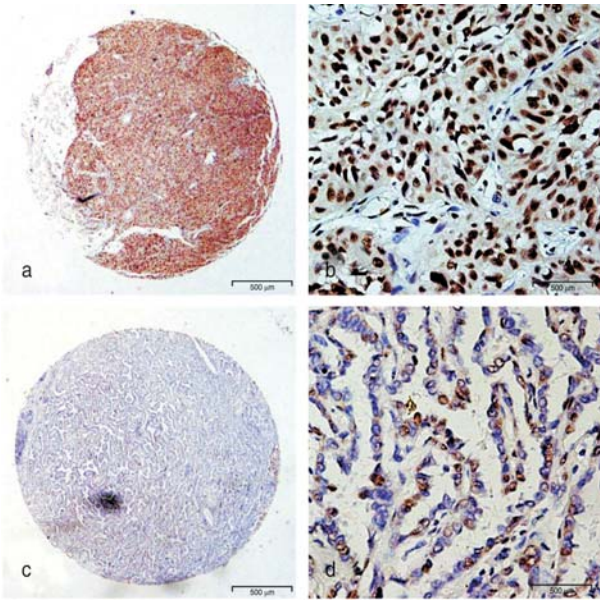

Fig. 2 Tissue microarray analysis of HIF-1 expression in lung cancer and noncancerous tissue. Immunohistochemical staining using anti-human HIF-1α antibody for detection of lung tissue HIF-1α expressions in different tissues from patients with lung cancer. (a and b) HIF-1α-positive expression in cytoplasm and cell membrane in cancerous tissue; (c and d) HIF-1α-negative expression, brown particles in the cytoplasm and cell membrane in the para-cancerous tissue. (a and c) S-P, original magnification × 40; (b and d) S-P, original magnification × 400

Table 2 The relationship between HIF-1α expression and clinicopathological features (n = 115)

Groups		No.	HIF-1α expression		χ^2	P
			Positive No.	%		
NSCLC		115	87	75.65		
Sex	Male	58	39	67.24	4.494	0.034
	Female	57	48	84.21		
Age (years)	≤ 60	53	40	75.47	0.002	0.967
	> 60	62	47	75.81		
Tumor diameter (cm)	≤ 2	97	77	79.38	4.679	0.031
	> 2	18	10	55.56		
Differentiation degree	Well	19	14	73.68	8.846	0.012
	Middle	84	68	80.95		
	Low	12	5	41.67		
Lymph node metastasis	Without	66	50	75.75	6.604	0.037
	Ipsilateral bronchial metastasis	43	35	81.40		
	Mediastinal	6	2	33.33		
TNM stage	I–II	16	13	77.36	0.169	0.919
	III–IV	99	74	75.00		
5-year survival	Survival	16	9	56.25	3.798	0.051
	Death	99	78	78.79		

located in the cytoplasm and cell nuclei in lung cancer tissues and the surrounding tissues. HIF-1α expression was high and uniform in cancerous tissue, increased in areas around the necrosis and tumor-infiltrating edge (Fig. 2a), and significant in tissues near the tumor edge (Fig. 2b). Furthermore, the lung cancer tissues displayed significantly higher levels of HIF-1α-positive expression, staining intensity, and IHC scores than the corresponding para-cancerous tissues (Fig. 2c and 2d).

Clinicopathological features of HIF-1α expression in lung cancer tissues

The clinicopathological features of HIF-1α expression in lung cancer tissues were shown in Table 2. The incidence of HIF-1α expression in lung tissues was 75.65% (87/115) in non-small cell lung cancer, and there were no significant correlations between positive HIF-1α expression and age, TNM stage, or 5-year survival rate. However, the clinicopathological features of HIF-1α expression in lung cancer tissues indicated a significant relationship between positive HIF-1α expression and patient sex (χ^2 = 4.494, P = 0.034), tumor size (χ^2 = 4.679, P = 0.031), differentiation degree (χ^2 = 8.846, P = 0.012) or presence of lymphatic node metastasis (χ^2 = 6.604, P = 0.037).

Discussion

The factors affecting the prognosis of lung cancer are complicated and directly related to inconspicuous early symptoms, technical defection in early diagnosis, and low awareness among patients. Often, lung cancer is definitively diagnosed in the middle or advanced stages, and the traditional treatments at these stages are surgical resection, chemotherapy, or radiotherapy [17]. Angiogenesis is a fundamental process involving a variety of pathological processes and sustains the progression of many neoplastic diseases. Moreover, it may enhance tumor cell proliferation and resistance to apoptosis, and facilitate metastasis. Tumor vasculature originates because of angiogenesis, vascular sheath growth, and endothelial progenitor cell growth. In the absence of vascularization, cell hypoxia causes tumor cells and macrophages to produce a large number of angiogenic factors that induce angiogenesis, which is important for the growth and development of lung cancer [18–21]. HIF-1α is suggested to be an important upstream molecule mediating VEGF expression and angiogenesis, and HIF-1α polymorphisms are reportedly associated with susceptibility to lung cancer [22–23]. In this study, we analyzed the expression of HIF-1α and changes in its expression in lung cancer tissues and peripheral blood by measuring the expression at the gene transcription or protein level.

The progress of lung cancer is closely related to the microenvironment and formation of new blood vessels. When lung cancer cells proliferate, tumor volume and oxygen consumption increase significantly, the cancer cells become hypoxic and overexpress HIF-1α, which leads to the secretion of angiogenic factors and induces angiogenesis. In the current study, we analyzed the expression of HIF-1α and VEGF in patients with benign and malignant lung disease. With a boundary of 100.0 μg/L for serum HIF-1α levels, the positive rate was 90.4% in adenocarcinoma, patients with pneumonia and normal groups had no abnormal; with a boundary serum VEGF level of 280.0 μg/L, the positive rate was 87% in

lung cancer patients and 13.3% in pneumonia patients. Furthermore, as the expressions of HIF-1α and VEGF were significantly increased and positively correlated, they can be considered as serological markers that reflect the progress of lung cancer and may be helpful in its diagnosis. IHC showed that the brown particles that represent HIF-1α expression in lung cancer and its surrounding tissues were located in the cytoplasm and cell nuclei, and HIF-1α-positive expression and intensity in the lung cancer group were significantly higher compared with those in the noncancer groups [24–26]. In addition, HIF-1α expression was significantly related with the sex, tumor size, differentiation degree, and lymph node metastasis; however, there was no significant correlation between HIF-1α expression and the age, TNM stage, and 5-year survival rate. Thus, HIF-1α may be a useful target for lung cancer therapy.

In summary, the study of the expression and change in expression of HIF-1α in human lung cancer tissues and peripheral blood by analyzing the transcription and translation level suggests that overexpression of HIF-1α regulates VEGF transcription and angiogenesis via a positive feedback mechanism, and an increase in the HIF-1α concentration can indicate an occurrence and development of cancer [27–30]. Detection of abnormal expressions of both HIF-1α and VEGF in lung cancer tissues, blood, and serum could indicate lung cancer and may be used for its diagnosis [31–33]. Importantly, inhibiting HIF-1α expression and increasing the binding of VEGF to the receptor could decrease the proliferation of vascular endothelial cells and contribute to the treatment of lung cancer.

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