Expression of Nerve Growth Factor and Hypoxia Inducible Factor-1α and Its Correlation with Angiogenesis in Non-Small Cell Lung Cancer*

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Summary: In order to investigate the expression of nerve growth factor (NGF) and hypoxia inducible factor-1α (HIF-1α) and its correlation with angiogenesis in non-small cell lung cancer (NSCLC), paraffin-embedded tissue blocks from 20 patients with NSCLC were examined. Twenty corresponding para-cancerous lung tissue specimens were obtained to serve as a control. The expression of NGF, HIF-1α, and vascular endothelial growth factor (VEGF) in the NSCLC tissues was detected by using immunohistochemistry. The microvascular density (MVD) was determined by CD31 staining. The results showed that the expression levels of NGF, HIF-1α and VEGF in the NSCLC tissues were remarkably higher than those in the para-cancerous lung tissues (P<0.05). There was significant difference in the MVD between the NSCLC tissues (9.19±1.43) and para-cancerous lung tissues (2.23±1.19) (P<0.05). There were positive correlations between NGF and VEGF, between HIF-1α and VEGF, and between NGF and HIF-1α in NSCLC tissues, with the spearman correlation coefficient being 0.588, 0.519 and 0.588, respectively. In NSCLC tissues, the MVD had a positive correlation with the three factors (P<0.05). Theses results suggest that NGF and HIF-1α are synergically involved in the angiogenesis of NSCLC.

Key words: non-small cell lung cancer; immunohistochemistry; nerve growth factor; hypoxia inducible factor-1α; vascular endothelial growth factor; CD31; microvascular density

The morbidity and mortality of lung cancer rank first in all malignant tumors[1]. About 85% of lung cancers are non-small cell lung cancer (NSCLC)[2]. Angiogenesis plays an important role in the occurrence, progression and metastasis of tumors, including NSCLC. Although the mechanism of angiogenesis in NSCLC has been extensively examined, it remains elusive.

It is well-known that neurotrophin and its receptor exert their significant roles in neurogenesis and neuroprotection. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are the main members that can stimulate the survival, differentiation and function of neural cells[3]. Most recent studies are focused on NGF, which exerts its biological effects by binding to its two receptors—a high affinity receptor tyrosine kinase receptor (TrkA) and a low affinity receptor (P75)[4]. In recent years, ample evidence shows that NGF is associated with vascular biology *in vitro*. It can increase the levels of vascular endothelial growth factor (VEGF) in normal neural cells and stimulate angiogenesis in the limb ischemia model[5, 6]. And similar results were also found in chick embryo chorioallantoic membrane (CAM), the most popular model for researching angiogenesis[7]. Moreover, in many non-neuronal tumors, including thyroid cancer, breast cancer, prostate cancer and lung cancer, etc., NGF is over-expressed and also can boost angiogenesis[8, 9].

Hypoxia inducible factor-1 (HIF-1) is an important factor produced by tumor tissues under hypoxia[10, 11]. Moreover, it has been identified to be involved in the development of many tumors, such as breast cancer, cervical cancer, osteosarcoma, lung and gastric carcinomas, etc.[12]. HIF-1 is a kind of dimer composed of α subunit and β subunits[13], and the former features HIF-1. Hypoxia *in vivo* can result in the up-regulation of HIF-1 expression. Subsequently, HIF-1α mediates the transcription of endogenous target genes (iNOS, ET-1, VEGF, EPO, etc.) on its downstream via hypoxia response element (HRE) to maintain oxygen homeostasis *in vivo*[14, 15].

VEGF, an important factor in angiogenesis, can not only integrate endothelial cells specifically and promote their growth, but also increase the vascular permeability of new vessels. It was revealed that VEGF is one of the target genes of NGF, and so does HIF-1[16, 17].

Nakamura *et al* reported that NGF can induce the VEGF expression via participation of HIF-1α in neuroblastoma[18]. Moreover, NGF and HIF-1α were found to induce the expression of VEGF and promote angiogenesis in a variety of tumor tissues. However, little

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is known about the association of NGF and HIF-1α in the angiogenesis of NSCLC. In order to identify their relationship, the expression of NGF and HIF-1α in NSCLC tissues and para-cancerous lung tissues was investigated in this study.

1 MATERIALS AND METHODS

1.1 Subjects

All the specimens were obtained from 20 patients with NSCLC aged 22–75 years (53.70±11.81 years), including 13 males and 7 females who underwent operation at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (HUST, China) from Jan. 2012 to Jan. 2013. The diagnoses of NSCLC were confirmed histopathologically. Each patient had complete clinical records. None of the patients in this study had received any chemotherapy or radiotherapy. According to the staging system of Union for International Cancer Control (UICC) (2009 revised), there were 9 cases at stage Ⅰ–Ⅱ and 11 at Ⅲ–Ⅳ. Based on standard pathological criteria, there were 9 cases of squamous cell carcinoma, 9 cases of adenocarcinoma and 2 cases of large cell carcinoma. Control tissue specimens were taken from the corresponding para-cancerous lung tissues more than 2 cm away from the tumor lesions. This research was conducted under the ethical approval of the Research Ethics Committee, Tongji Medical College, HUST (China).

1.2 Immunohistochemistry

Each paraffin-embedded tissue specimens was cut into four 4-μm-thick serial sections, and negative control specimens were prepared as well. For immunohistochemistry (IHC), rabbit anti-human NGF polyclonal antibody, rabbit anti-human HIF-1α polyclonal antibody, rabbit anti-human VEGF polyclonal antibody and rabbit anti-human CD31 monoclonal antibody (Wuhan Boster Co., China) were used as primary antibodies, and normal goat serum as a negative control. After deparaffinization, hydration and incubation with 3% hydrogen peroxide for 30 min, the slices were immersed into citric acid solution (0.01 mol/L) for antigen retrieval for 20 min at 92–94°C. Then, the sections were incubated with following primary antibodies (rabbit anti-human NGF polyclonal antibody and rabbit anti-human VEGF polyclonal antibody, 1:150 dilution; rabbit anti-human HIF-1α polyclonal antibody and rabbit anti-human CD31 monoclonal antibody, 1:100 dilution) overnight at 4°C. The samples were then incubated with biotinylated goat-anti-rabbit IgG for 20 min at room temperature. After incubation with horseradish peroxidase for 30 min, they were exposed to DAB (Wuhan Boster Co., China) solution according to the manufacturer's protocol and counterstained with hematoxylin.

1.3 Evaluation of Results

1.3.1 NGF, HIF-1α and VEGF For each section, the percentage and the staining intensity of positive cells were determined. Each section was scored based on the percentage and the staining intensity of positive cells counted in 10 randomly selected fields. The percentage of positive cells was scored as follows: 0, <5%; 1, 5%–24%; 2, 25%–50%; 3, >50% positive cells. And the staining intensity of positive cells was scored as follows: 0, none; 1, light yellow; 2, tan; 3, brown. According to the sum score of each section, two groups were set up: low expression group (≤3) and high expression group (>3). Scoring was performed by two observers.

1.3.2 Microvascular density (MVD) An independent unit of capillaries is any brown-stained endothelial cell or endothelial cell cluster, which is obviously different from tumor cells and mesenchyme components around. Positive staining of independent units of capillaries, so-called "hot spots", was counted as MVD in 10 randomly selected high-powered fields, and the result was recorded as $\bar{x}$±s.

1.4 Statistical Analysis

Statistical analysis was performed using SPSS17.0 program (SPSS, USA). The results were analyzed by chi-square test, correction for chi-square test, Fisher probabilities and two-group *t*-test. A *P* value less than 0.05 was considered to be statistically significant.

2 RESULTS

2.1 Expression of NGF, HIF-1α and VEGF, and MVD in NSCLC Tissues and Para-cancerous Lung Tissues

The cell membrane and cytoplasm were stained brown in cells positive for NGF, HIF-1α or VEGF. The expression levels of NGF, HIF-1α and VEGF in NSCLC tissues were remarkably higher than those in para-cancerous lung tissues (P<0.05). Moreover, there was significant difference in the MVD between NSCLC tissues (9.19±1.43) and para-cancerous lung tissues (2.23±1.19) (P<0.05) (table 1, fig. 1).

Table 1 Expression of NGF, HIF-1α and VEGF, and MVD in NSCLC tissues and para-cancerous lung tissues

Groups	*n*	NGF		HIF-1α		VEGF		MVD
		High	Low	High	Low	High	Low	($\bar{x}$±*s*)
NSCLC tissues	20	15	5	17	3	17	3	9.19±1.43
Para-cancerous lung tissues	20	4	16	4	16	2	18	2.23±1.19
P		0.002*		0.000*		0.000*		0.000*

*P<0.05

2.2 Correlation of NGF, HIF-1α and VEGF Expression in NSCLC Tissues

Statistical analysis demonstrated that in NSCLC tissues, NGF expression was related to HIF-1α expression (r=0.588) and VEGF expression (r=0.588), respectively. Additionally, significant relationship was noted between HIF-1α expression and VEGF expression (r=0.519, table 2).

2.3 Correlation of NGF, HIF-1α and VEGF Expression with MVD in NSCLC Tissues

As statistical analysis demonstrated, the expression levels of NGF, HIF-1α and VEGF were significantly

correlated with the MVD in NSCLC tissues (P<0.05, table 3).

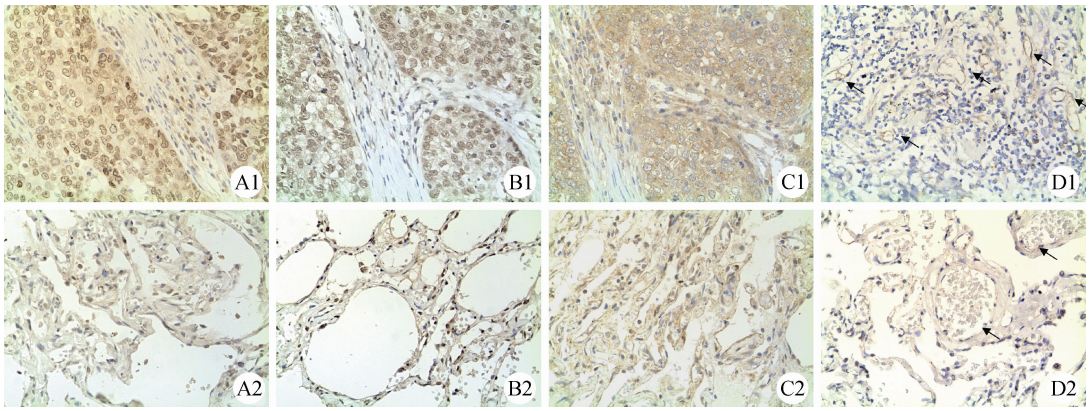

Fig. 1 Immunohistochemical staining of NGF, HIF-1α, VEGF and MVD in NSCLC tissues and para-cancerous lung tissues (from the same source, SABC, ×200)

The expression of NGF in NSCLC tissues (A1) was increased as compared with that in the para-cancerous lung tissues (A2). HIF-1α in NSCLC tissues (B1) was highly expressed as compared with that in para-cancerous lung tissues (B2). In NSCLC tissues (C1), VEGF expression was higher in NSCLC tissues than in para-cancerous lung tissues (C2). D1 and D2 indicated the MVD in NSCLC tissues and para-cancerous lung tissues respectively. Arrows indicated the MVD in lung tissues.

Table 2 Correlation of NGF, HIF-1α and VEGF expression in NSCLC tissues

	NGF and VEGF	HIF-1α and VEGF	NGF and HIF-1α
r	0.588	0.519	0.588
P	0.009*	0.001*	0.009*

*P<0.05

Table 3 Correlation between NGF, HIF-1α and VEGF expressions with the MVD in NSCLC tissues

MVD	n	NGF, HIF-1α and VEGF expression				P
		Co-expression of three	Co-expression of two	Expression of one	Non-expression of three	
High MVD (≥9.19)	14	13	0	1	0	
Low MVD (<9.19)	6	2	1	1	2	0.025*

*P<0.05

3 DISCUSSION

In this study, we found that NGF and HIF-1α were highly expressed in NSCLC tissues as compared with para-cancerous lung tissues. The expression of NGF and HIF-1α was closely correlated with the MVD in NSCLC. The results suggested that NGF and HIF-1α may play important roles in the angiogenesis of NSCLC, which were consistent with previous findings that over-expressed NGF and HIF-1α can boost angiogenesis in tumor tissues[8, 14].

Previous studies showed that NGF could boost angiogenesis in breast cancer and ovarian carcinoma tissues[16, 19]. Some findings reported that TrkA, a high affinity receptor of NGF, was over-expressed in lung cancer[20]. K252a, the TrkA receptor inhibitor, could obviously inhibit the growth of lung adenocarcinoma cells *in vitro*[20]. Moreover, various researches reported that TrkA receptors could promote the expression of VEGF, the best-known angiogenic stimulator, in most tissues[21]. However, it is unclear about the precise mechanism underlying the associations among NGF, TrkA and VEGF. Previous reports indicated that various signaling pathways were involved in the effect of TrkA on VEGF, of which PI3K-Akt pathway was extensively mentioned[22]. Moreover, it was reported that NGF can induce the VEGF production in various tissues[21, 23]. Our results also showed that the NGF expression was closely correlated with the expression of VEGF.

As various researches reported, HIF-1α, the important factor in hypoxia, is implicated in VEGF production. It can mediate the VEGF expression in several tissues or cells, and eventually promote angiogenesis[24–26]. Our research also showed that the increase in HIF-1α expression was accompanied by the increase in VEGF production in NSCLC.

Recently, Nakamura *et al*[18] reported that NGF-induced VEGF transcription was dependent on the increased production of HIF-1α in neuroblastoma. In their study, it was found that pharmacologic inhibitors of the Trk tyrosine kinase, PI-3 kinase and mTOR pathways prevented NGF stimulated increases in HIF-1α and VEGF. Similarly, Kim *et al*[27] found that TrkA activation on NGF led to VEGF elevation via PI3K-Akt pathway and therefore suggested that TrkA could be a stimulator of retinal vascular development. HIF-1 was reported to be a transcriptional activator of TrkA[28]. Conserved HRE sequences, (A/G) CGTG, was found on the regulatory region of 5' upstream of NTRK1 gene (gene of TrkA) by bioinformatics analyses. All these findings revealed an intrinsic relationship among NGF, TrkA, HIF-1α and VEGF.

The results in our study lend support to the

conclusion that NGF induces increases in VEGF expression via HIF-1α participation. It was suggested that there might be a certain synergy among them in the angiogenesis of NSCLC tissues. We speculated under hypoxia caused by the uncontrolled growth of NSCLC cells, the NGF expression increased, activated its receptor TrkA and induced VEGF production, which eventually promoted angiogenesis. HIF-1α participated in the whole procedure.

In conclusion, our study revealed that NGF and HIF-1α are over-expressed in NSCLC tissues. They are synergically involved in the angiogenesis of NSCLC. However, the relationship between NGF and VEGF remains to be clearly elucidated. Additionally, studies on the participation of HIF-1α in TrkA signaling need to be conducted. The implication of NGF and HIF-1α in tumor angiogenesis is expected to become a new hotspot of neural and vascular researches, and it will provide a novel strategy for molecular targeted therapy in tumors.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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