RESEARCH ARTICLE

Expression of Hypoxia-inducible Factor Prolyl Hydroxylase 3 HIFPH3 in Human Non-small Cell Lung Cancer (NSCLC) and Its Correlation with Prognosis

Xiao Chu[1], Cheng-Chu Zhu[2], Hui Liu[1]*, Jiao-Chen Wang[3]*

Abstract

Purpose: To investigate the expression of hypoxia-inducible factor prolyl hydroxylase 3 (HIFPH3) in non-small cell lung cancer (NSCLC) and explore the correlation of HIFPH3 expression with lymph node metastasis and microvessel density (MVD). Materials and Methods: A total of 73 cases of NSCLC specimens, 24 cases of para-cancerous tissues, and 20 normal pulmonary tissues were collected for HIFPH3 and CD31 immunohistochmical (IHC) study. Microvessel density (MVD) of the NSCLC tissues was also determined based on the expression of CD31. Results: The expression of HIFPH3 in carcinoma tissue was statistically higher than para-cancerous and normal pulmonary tissues (χ^2=48.806, p<0.05). Compared withthe negative lymph node metastasis group, the lymph node metastasis group showed significantly higher HIFPH3 expression (χ^2=6.300, p<0.05). The strong HIFPH3+group displayed a significantly higher MVD than weak HIFPH3+ and HIFPH3- groups (p<0.05). No differences in positive HIFPH3 expression were noted regarding the tumor diameter, age, smoking status, gender of NSCLC patients, tumor size, histopathology, or differentiation. Conclusions: HIFPH3 expression in human NSCLC lesions is significantly higher than that in para-cancerous and normal lung tissues and is positively associated with lymph node metastasis and MVD.

Keywords: NSCLC - hypoxia-inducible factor prolyl hydroxylase 3 - CD31 - microvessel density

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Introduction

Lung cancer is becoming the worldwide top male cancer type with highest morbidity and mortality (Coté et al., 2012). About 80-85% lung cancer cases are none-small cell lung cancer (NSCLC). Despite of growing diagnostic technologies, more than 70% lung cancer patients were first diagnosed as intermediate or late stage. Currently, the main therapeutic method for lung cancer is operation combined with radiotherapy and chemotherapy, to which only 20% patients are sensitive with a median survival time of 10 months (Schiller et al., 2002). There is no significant change for prognosis of lung cancer patients during the past two to three decades.

In most solid tumors, cancer cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a low rate of glycolysis followed by pyruvate oxidation in mitochondria even with the presence of plenty oxygen. This is called Warburg effect (Samudio et al., 2009). The adaption of cancer cells to hypoxia is not only essential for tumor growth and development (Pouysségur et al., 2006), but also accounts for radio- and chemo-therapy tolerance (Brown, 2002; Brahimi-Horn et al., 2007; Vaupel, 2008). Hypoxia keeps cells from differentiation and promotes blood vessels formation, in which hypoxia-inducible factor-1 (HIF-1) is functionally important. Induced by hypoxia, HIF1 is a hetero-dimer basic helix-loop-helix (bHLH) transcription factor composing of α and β subunit. There are three types of HIF-β, with constitutive expression level in nucleus. HIF-α family contains three members, among which HIF-1α and HIF-2α are the main functional members (Maxwell et al., 1993; Ema et al., 1997; Semenza, 2003; Li et al., 2006; Li et al., 2013a). The HIF signaling cascade mediates the effects of hypoxia, including blood vessels formation, tumor invasion, and metastasis (Li et al., 2013b; Zhang et al., 2014).

The proline residues of HIF-α could be hydroxylated by hypoxia-inducible factor prolyl hydroxylases (HIFPHs). The protein level of hydroxylated HIF-α is regulated by 26S proteasome through recruiting pVHL, Elongin C, Elongin B, Cul22, and Rbx1 to form E3 ubiquitin ligase complex (Min et al., 2002). Among the three human HIFPHs, HIFPH2 has the strongest hydroxylation towards HIF-1α (Appelhoff et al., 2004). As the cell oxygen sensor, HIFPH activity is modified by oxygen concentration. With lower than 20% oxygen concentration, HIFPH activity becomes decreasing, resulting in reduced ubiquitination

[1]*Department of Thoracic Surgery, The Fifth People's Hospital of Shanghai, Fudan University, Shanghai,* [2]*Department of Thoracic Surgery,* [3]*Department of Pathology, Taizhou Hospital of Zhejiang Province, Linhai , China *For correspondence: jiaochenwangjcw@163.com, huiliuhl@163.com*

mediated HIF-α degradation and accumulated HIF-α level, which in turn could induce the expression of HIFPH2-3. With the presence of relatively high oxygen concentration, HIFPH level is high and the HIF-α level is kept at certain level due to the active proteasome mediated HIF-α degradation (D'Angelo et al., 2003). HIFPH2 is active under normal oxygen or mild hypoxia, while HIFPH3 is the main regulator of HIF-α under severe hypoxia or long term hypoxia (AppelhoffTian et al., 2004). The expression detection of HIF and associated target genes is widely used to indicate the relationship of tumor hypoxia status and prognosis, while there are limited reports on HIFPHs.

In this study, we detected the HIFPH3 expression in NSCLC using immunohistochemical (IHC) method. We also counted the microvessel density (MVD) in lung tumor and explored the relationship between HIFPH3 expression and prognosis of NSCLC patients.

Materials and Methods

Specimens

The 73 cases of paraffin sections (46 males and 27 females; average age 58.3 years, from 35-80 years) were from the Human Tissue Specimen Bank, Zhejiang Taizhou Hospital affiliated to Wenzhou Medical College. The specimens were obtained from operation excised tissue of NSCLC patients during Mar. 2004-Dec. 2010 [Lobectomy plus lymphadenectomy (n=54), whole lung resection with lymphadenectomy (n=14), pulmonary bump and nodule resection (n=4), partial tumor resection (n=1)]. Meanwhile, 24 cases of para-cancerous tissue (P, 1.5cm from tumor) and 20 cases of normal pulmonary tissue adjacent to tumors (N, 5cm from tumor) were excised as experimental controls. All the patients received no chemo- or radio-therapy before the surgery. All the samples were fixed with 10% formaldehyde solution and embedded with paraffin. All the samples were collected upon the agreement of patients and experiments were approved by Local Ethnic Committee.

IHC

Hematoxylin-eosin (H&E) staining and IHC were performed on 4-5μM sections of formalin fixed, paraffin embedded tumors. Following deparaffinization and rehydration of the tissue sections, antigen retrieval was performed at high temperature and high pressure in 10 mM citrate buffer (pH6) or using microwave treatment in Tris-EDTA buffer. After serum incubation, the tissue sections were applied with primary antibody. Primary HIFPH3 antibody (Abcam, MA, USA) and CD31 antibody (Life Technologies, NY, USA) were applied at 1:200 and 1:25 dilutions, respectively. The second antibody of A solution (ChemMate TM Envision+/HRP, kit from Envision) was applied to the tissue sections for primary antibody recognition. Staining development was achieved by incubation with DAB (Shanghai Gene Tech Company Limited, Shanghai, China). The positive control sections were affiliated in the Envision kit. The positive control for HIFPH3 and CD31 were placenta and blood vessel epithelium, respectively. For negative controls were the staining results of the respective tissue sections incubated with TBS instead of primary antibody. The HIFPH3 and CD31 IHC scoring was conducted with double-blind method. Under high power fields (HPF), 5 fields were randomly selected and the positive rate was calculated as the positive stained cell number in 400 cells. Cells with yellow or pale brown cytoplasm were HIFPH3+, while cells with yellow, pale brown cell membrane were CD31+. The IHC scoring was referred to the method developed by Fields et al. (Shijubo et al., 1999; Fields et al., 2004). The scoring method and grading criteria are listed in (Table 1). The MVD was calculated based on CD31 staining (Weidner et al., 1992). Each positive endothelial cell cluster of immunoreactivity in contact with the selected field was counted as an individual vessel in addition to the morphologically identifiable vessels with a lumen. The average microvessel number of 5 HPFs (200×) was calculated as MVD. Macrophages and plasma cells with positive signals were excluded based on their morphologies.

Statistical Analysis

All the data analysis was performed with SPSS 13.0 (SPSS Inc, Chicago, IL, USA). The HIFPH3 and CD31 expression, and the relationship between HIFPH3 expression and the clinic pathological factors of NSCLC were conducted using Chi-squared test, corrected Chi-squared test, exact probability method, and Spearman rank correlation analysis. The MVD comparisons among groups were performed with group t-test method. $p<0.05$ was designated as significant difference.

Table 1. Method and Standard for HIFPH3 IHC Scoring

Percentage of Positive Cell	No staining (0)	Light yellow (1)	Pale brown (2)
≤5% (0)	0 (-)	0 (-)	0 (-)
5%≤25% (1)	0 (-)	1 (-)	2 (+)
25%≤50% (2)	0 (-)	2 (+)	4 (++)
>50% (3)	0 (-)	3 (+)	6 (++)

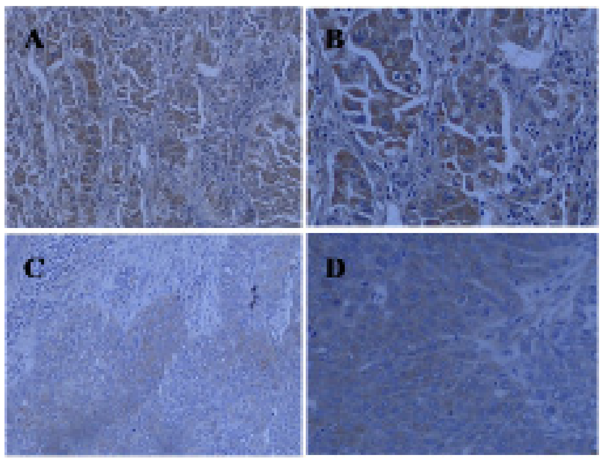

Figure 1. The IHC Staining of HIFPH3 in NSCLC. (A-B) positive HIFPH3 IHC staining in adenocarcinoma cells (A) SP × 100; (B) SP × 400); (C-D) positive HIFPH3 IHC staining in squamous-cell carcinoma cells (C) SP×100; (D) SP × 400)

Table 2. Expression of HIFPH3 Protein in Different Lung Tissues

Group	Sample (N)	HIFPH3 expression (N)			χ^2	*P* value
		−	+	++		
Lung cancer tissue	73	28	26	19	48.806	0
Para-cancerous tissue	24	22	2	0		
Normal pulmonary tissue	20	20	0	0		

Table 3. Relationship Between HIFPH3 Protein Expression and Clinic Pathological Characteristics in NSCLC

Variables	Sample (N)	HIFPH3 expression (N)			χ^2	*P*
		−	+	++		
Age (years)						
≥55y	49	19	17	13	0.057	0.972
<55y	24	9	9	6		
Gender						
Male	46	16	18	12	0.845	0.665
Female	27	12	8	7		
Smoking history						
Ever	48	16	19	13	1.601	0.449
Never	25	12	7	6		
Tumor size (cm)						
≤3	29	13	11	5	2.025	0.363
>3	44	15	15	14		
Pathological type						
Squamous-cell carcinoma	39	15	14	10	0.007	0.997
Adenocarcinoma	34	13	12	9		
Tissue differentiation						
Un-/Poorly differentiation	22	6	11	5	2.969	0.227
Moderately / Well differentiation	51	22	15	14		
Lymph node metastasis						
Negative lymph node metastasis	39	20	12	7	6.3	0.043
Positive lymph node metastasis	34	8	14	12		

Results

HIFPH3 expression in NSCLC

No HIFHP3 IHC staining was observed in all the negative controls. In IHC positive samples, HIFHP3 IHC staining was detected in cytoplasm as yellow and brown-yellow (Figure 1). The summary of expression of HIFPH3 in different pulmonary tissues is listed in (Table 2). The positive rate of HIFPH3 was 61.6% (45/73), of which there were 26 weak HIFPH3+cases and19 strong HIFPH3+cases. The positive rate of HIFPH3 in para-cancerous tissue and normal pulmonary tissue were 8.3% (2/24) and 0 (0/20), respectively. The expression of HIFPH3 in carcinoma tissue was statistically higher than that of para-cancerous and normal pulmonary tissues (χ^2=48.806, p<0.05).

For the 34 lymph node metastasis cases among the 73 NSCLC samples, the positive rate of HIFPH3 was 76.5% (26/34), of which there were 14 weak HIFPH3+cases and12 strong HIFPH3+cases (Table 3). In the negative lymph node metastasis group, the positive rate of HIFPH3 was 48.7% (19/39), of which there were 12 weak HIFPH3+cases and 7 strong HIFPH3+cases (Table 3). The expression of HIFPH3 in lymph node metastasis group was statistically higher than that in negative lymph node metastasis group (χ^2=6.300, p<0.05). There were no differences of positive expression of HIFPH3 regarding to the tumor diameter, age, smoking status, gender of NSCLC patients, tumor size, histopathology, or differentiation (P >0.05) (Table 3).

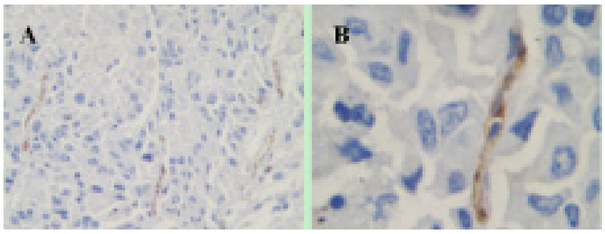

Figure 2. IHC Saining of CD31 in Microvessel of Non-small Cell Lung Cancer. (A) SP×100; (B) SP×400.

Table 4. Relationship Between HIFPH3 Protein Expression and MVD in NSCLC

HIFPH3 expression	Sample (N)	MVD	*P*
−	28	31.67±5.15	< 0.05
+	26	35.37±5.57	< 0.05
++	19	40.32±6.91	< 0.05

Positive correlation of HIFPH3 expression with MVD in NSCLC

The CD31 IHC staining results showed that CD31 was located in the cytoplasm of the vascular endothelial cells. The CD31 IHC staining signal was yellow and brown-yellow (Figure 2). The MVDs for HIFPH3- cases, weak HIFPH3+cases and strong HIFPH3+cases were 31.67±5.15, 35.65±5.57, and 40.32±6.91, respectively (Table 4). The expression of HIFPH3 was positively correlated with MVD. The MVD in strong HIFPH3+group was significantly higher than those in weak HIFPH3+and HIFPH3- group (p<0.05) (Table 4).

Discussion

Although normal pulmonary epithelium is accessible to relatively high concentration of oxygen, the human NSCLC tissue is in the hypoxia status (average oxygen pressure 2.2%, ranging from 0.1%-6%) (Swinson et al., 2003; Le et al., 2006). HIF-1α and its regulator HIFPH3 are important factors involved in the cellular response to hypoxia. In this study, we detected the expression of HIFPH3 and the microvessel marker CD31 in NSCLC samples via IHC. We also explored the relationship between HIFPH3 expression and the clinic pathological factors of NSCLC.

The expression of HIFPH3 (both protein and mRNA) in human NSCLC lesions were significantly higher than those in para-cancerous and normal lung tissues, which is consistent with previous reports in pancreatic endocrine tumors and renal cell carcinoma (Raval et al., 2005;

Couvelard et al., 2008). And HIFPH3 expression were positively correlated with lymph node metastasis, which is also consistent with the results from Couvelard et al. (RavalLau et al., 2005; CouvelardDeschamps et al., 2008). Hypoxia could disrupt tissue integrity through repression of E-cadherin expression (Fu et al., 2014). Hypoxia could also promote proteolytic activity at the invasive front and alter the interactions between integrins and components of the extracellular matrix (Nishii et al., 2013). The high HIFPH3 expression in NSCLC could facilitate metastasis by regulating HIF-1α, resulting in hypoxia responses in tumor.

CD31 is found on the surface of platelets, monocytes, neutrophils, and some types of T-cells, and makes up a large portion of endothelial cell intercellular junctions (Albelda et al., 1991; Dejana, 2004). CD31 is involved in leukocyte migration (Muller, 1995), angiogenesis (DeLisser et al., 1997), and integrin activation (Bussolino et al., 1997; Reedquist et al., 2000). As a microvessel marker, CD31 is utilized to determine MVD. We found that the MVD in strong HIFPH3+was significantly higher than those in weak HIFPH3+and HIFPH3- group. Angiogenesis and lymphangiogenesis provides the necessary routes for dissemination. VEGF-induced changes in vascular integrity and permeability promote both intravasation and extravasation, while VEGF-induced angiogenesis in the secondary tissue is essential for cell proliferation and establishment of metastatic lesions (Stacker et al., 2002; Choi et al., 2005; Ma et al., 2014). Although tumor tissue tends to acquire more oxygen and nutrition through angiogenesis, it remains in hypoxia status due to vigorous growth. Our results indicates that high HIFPH3 expression level is closely related to the tumor hypoxia status, while further investigations are needed to unveil the molecular mechanism.

For statistical analysis, the sample size was relatively small in our study. In order to get much more solid conclusions, NSCLC samples from different hospitals in different areas could be combined to form big sample size. Our study is pure HIFPH3 expression observation and correlation results is merely indirect evidence when regarding to mechanism exploration. More functional studies are needed. For example, whether conditional HIFPH3 knock out or knock down in mouse NSCLC model could inhibit tumor growth or metastasis.

In conclusion, through HIFPH3 IHC staining and various statistical analysis, we found that the expressions of HIFPH3 in human NSCLC lesions were higher than those in para-cancerous and normal lung tissues, and high HIFPH3 expression was positively associated with lymph node metastasis. MVD in strong HIFPH3+high positive group was significantly higher than those in weak HIFPH3+and HIFPH3- group. Our results provided important evidences to unveil the functions of HIFPH3 in tumor hypoxia response. Our research also pointed out the possibility that HIFPH3 can be served as NSCLC marker for prognosis and potential therapeutic target.

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