RESEARCH COMMUNICATION

Protein Expression and Significance of VEGF, EGFR and MMP-9 in Non-Small Cell Lung Carcinomas

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Abstract

Objective: This study was designed to detect the protein expression of VEGF, EGFR and MMP-9, to investigate the potential roles they might play in the pathogenesis of NSCLC and to discuss their relationship and their clinical significance. Methods: For 136 cases who were diagnosed of NSCLC, immunohistochemistry was used to detect protein expression of VEGF, EGFR and MMP-9 tumour and normal lung tissue specimens. Statistical analysis was performed on the relationship between protein expression of VEGF, EGFR and MMP-9 and clinico-pathological parameters and prognosis. Results: Expression of VEGF and MMMP-9 was mostly in the cytoplasm, while EGFR wasfound in both cytoplasm and membranes. In NSCLCs, the positive rate of VEGF protein was 79.4% (108/136), for EGFR was 75.0% (102/136) and for MMP-9 was 68.4% (93/136), significantly greater than the normal tissue values of 16.0% (8/50), 12.0% (6/50) and 8.0% (4/50), respectively (P<0.01). Expression of VEGF, EGFR and MMP-9 was related to pathology grading, lymph node metastasis and clinical staging in NSCLC (P<0.01), while being independent of other clinicopathologic parameters (P>0.05). There was an obvious positive correlation among the expression of VEGF and EGFR (r=0.25; P<0.01), VEGF and MMP-9 (r=0.28; P<0.01), EGFR and MMP-9 (r=0.19; P<0.05) in NSCLCs. Conclusions: Protein expression of VEGF, EGFR and MMP-9 is elevated higher in NSCLC, correlating with progression.

Keywords: NSCLCs - VEGF - EGFR - MMP-9 -immunohistochemistry

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Introduction

Primary bronchogenic carcinoma of lung has become the highest mortality rate of malignant tumors in the world, among them, the proportion of non-small cell lung cancer (non small cell lung cancer, NSCLC) accounts 85%, most patients that have been already clinically diagnosed are in middle-advanced stage , and the 5 years survival rate is very low (Jemal et al., 2008). Thus, early diagnosis and early treatment are particularly important to improve the survival rate.

It is now considered that the evolution of NSCLC may involve the various pathogenic factors. One of the important factors is the formation of blood vessels in NSCLC, and vascular endothelial growth factor (VEGF) is the most potent known stimulator of angiogenesis (Kojima et al., 2005). Other factors which are colsely related with angiogenesis and genesis of NSCLC include epidermal growth factor receptor (EGFR), matrix metalloproteinase (MMP-9), and etc (Jeon et al., 2006, Safranek et al., 2009). The conjunct expression of them, the correlation and the roles in NSCLC have not been reported.This study used immunohistochemistry technique to investigate the expression of VEGF, EGFR, and MMP-9, to investigate interrelationship and to explore theirs relationship with clinical pathological features in NSCLC, so that the reference can be provided for diagnosing NSCLC and identifying new therapeutic targets.

Materials and Methods

Clinical data

Specimens were obtained from archived paraffin-embedded tissue sections of 136 patients with NSCLC at the the Third Affiliated Hospital, Sun Yat-sen University, Guangzhou from January 1st, 2009 to December 31st, 2010. A group of 50 normal lung tissue cases was conducted as control. There were 97 men and 39 women with a median age 60 years (range 30-82 years). According to the World Health Orgaization criteria of 2004 (Rami-Porta et al., 2009), NSCLC were classified as follows: adenocarcinoma: 72 cases, squamous cell carcinoma: 40 cases, other types: 24 cases. High-moderately differentiated carcinoma: 88 cases, poorly differentiated carcinoma: 48 cases. According to the Staging standard of the TNM system of International Association for the Study of Lung Cancer (Di et al., 1998), NSCLC were classified as stage I,II: 96 cases, and stage III, IV: 40 cases. Metastasis

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to lymph nodes was evident in 35 cases.

Protein expression of VEGF, EGFR and MMP-9 in NSCLC

Immunohistochemistry technique that uses the streptavidin-peroxidase(S-P) was employed for VEGF, EGFR and MMP-9 detection. Mouse anti-human monoclonal antibody of VEGF, EGFR,MMP-9 and SP kit were purchased from Maxim biological and technical company, Fuzhou, China; ready-to-use. All the sections were routinely deparaffinized and rehydrated, then the sections were rinsed in phosphate-buffered saline (PBS, pH=7.4), subsequently were treated for antigen retrieve. Sections were treated in EDTA buffer (pH = 8.0) in autoclave sterilizer. After cooling at room temperature for 20 min, the sections were rinsed in PBS, then immersed in 3% H_2O_2 for 15 min to block the endogenous enzymes. After being rinsed in PBS, the sections were incubated with normal goat serum at 37˚C for 15 min to block nonspecific antibodies. After interaction with VEGF antibody, EGFR and MMP-9 antibodies (monoclonal antibody), the sections were rinsed in PBS, then incubated with biotinylated secondary antibodies and rinsed in PBS again. After interaction with streptavidin-HRP and being rinsed in PBS, the sections were visualized by reaction with 3,3'-diaminobenzidine and counterstained with hematoxylin. They were then dehydrated, transparented and covered with coverslips and sealed with neutral gum.PBS substituting the primary antibody was used as negative control.

Judgement of positive result of VEGF, EGFR and MMP-9

The judgement of whether the tumor and the normal tissues were positive or not was performed by two pathology doctors. The positive expression of VEGF was mostly in cytoplasm with brown-yellow color, EGFR was mostly in cytoplasm and membrane with brown-yellow color, and MMP-9 was mostly in cytoplasm with brown-yellow color (see Figure 1). A tumor or normal tissue in which more than 10% of cells were stained with those antibodies was recognized as positive. Less than 10% of positive cells was recognized as negative.

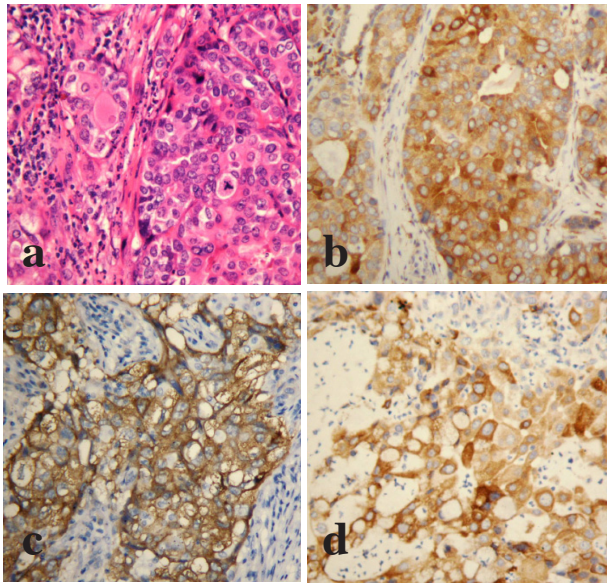

Figure 1. Sections of a Representative NSCLC, a) Haematoxylin and eosin x 200; b) VEGF in the cytoplasm; c) EGFR in cytoplasm and membranes; d) MMP-9 in cytoplasm; IHC x 200

Table 1. Clinical and Pathologic Characteristics of the 356 CRC Cases in this Study

Parameter	n	VEGF		EGFR		MMP-9	
Sex							
Male	97	78 (80.4)	>0.05	73 (75.3)	>0.05	65 (67.0)	>0.05
Female	39	30 (76.9)		29 (74.4)		28 (71.8)	
Age (years)							
60	61	47 (77.0)	>0.05	45 (73.8)	>0.05	43 (70.5)	>0.05
≥60	75	61 (81.3)		57 (76.0)		50 (66.7)	
Tumor size							
3	51	39 (76.5)	>0.05	37 (72.5)	>0.05	33 (64.7)	>0.05
≥3	85	69 (81.2)		65 (76.5)		60 (70.6)	
Lymph node metastasis							
Yes	45	43 (95.6)	<0.01	42 (93.3)	<0.01	40 (88.9)	<0.01
No	91	65 (71.4)		60 (65.9)		53 (58.2)	
Differentiation							
High	88	63 (71.6)	<0.01	59 (67.0)	<0.01	51 (58.0)	<0.01
Low	48	45 (93.8)		43 (89.6)		42 (87.5)	
Pathology typing							
SCC	40	31 (77.5)	>0.05	29 (72.5)	>0.05	27 (67.5)	>0.05
AC	72	57 (79.2)		55 (76.4)		49 (68.1)	
Other	24	20 (83.3)		18 (75.0)		17 (70.8)	
Tumor location							
Peri	60	46 (76.7)	>0.05	43 (71.7)	>0.05	39 (65.0)	>0.05
Central	76	62 (81.6)		59 (77.6)		4 (71.1)	
TNM stage							
I+II	96	70 (72.9)	<0.01	66 (68.8)	<0.01	59 (61.5)	<0.01
III+IV	40	38 (95.0)		36 (90.0)		34 (85.0)	

SCC, Squamous carcinoma; AC, Adenocarcinoma; Peri, peripheral

Statistical analysis

Data were analyzed with computer aided SPSS13.0 statistical software. Relationship between expression of VEGF, EGFR and MMP-9 and clinic pathological parameters was evaluated by the $\chi 2$ analyses. Correlation between two variables was evaluated by the Spearman rank correlation test. A value of $P<0.05$ was considered statistically significant

Results

Relationship between protein expression of VEGF and clinic pathological parameters in NSCLC

In NSCLCs, the expression of VEGF was mostly in cytoplasm. There were 108 cases with positive expression of VEGF in 136 cases of NSCLC, and negative cases were 28.The positive rate of VEGF was 79.4 (108/136) in NSCLC. The positive rate of VEGF was 16.0 (8/50) in normal lung tissue. There was significant difference of expression of VEGF between NSCLC and normal lung tissue ($P<0.01$). The expression of VEGF was related to differentiation degree, lymph node metastasis and clinical Staging in NSCLC ($P<0.01$), while independent of other clinicopathologic parameters ($P>0.05$)(Table 1).

Relationship between protein expression of EGFR and clinic pathological parameters in NSCLC

The expression of EGFR was mostly in membrane or

cytoplasm. There were 102 cases with positive expression of EGFR in 136 cases of NSCLC, and negative cases were 34.The positive rate of EGFR was 75.0 (102/136) in NSCLC. The positive rate of EGFR was 12.0 (6/50) in normal lung tissue. There was significant difference of expression of EGFR between NSCLC and normal lung tissue (P<0.01). The expression of EGFR was related to differentiation degree, lymph node metastasis and clinical Staging in NSCLC (P<0.01), while independent of other clinicopathologic parameters (P>0.05) (Table 1).

Relationship between protein expression of MMP-9 and clinic pathological parameters in NSCLC

The expression of MMP-9 was mostly in cytoplasm. There were 93 cases with positive expression of MMP-9 in 136 cases of NSCLC, and negative cases were 43.The positive rate of MMP-9 was 68.4 (93/136)i n NSCLC. The positive rate of VEGF protein was 8.0 (4/50)in benign hepatic tissue. There was significant difference of expression of MMP-9 between NSCLC and normal lung tissue (P<0.01). The expression of MMP-9 was related to differentiation degree, lymph node metastasis and clinical Staging in NSCLC (P<0.01), while independent of other clinicopathologic parameters (P>0.05) (Table 1).

Correlation of protein expression of VEGF, EGFR and MMP-9 in NSCLCs

Spearman rank correlation analysis showed that protein expression of VEGF and EGFR was associated, and was positively correlated (r=0.25, P<0.01) in NSCLC. Protein expression of VEGF and MMP-9 was positively correlated(r=0.28, P<0.01). Protein expression of EGFR and MMP-9 was also positively correlated (r = 0.19, P <0.05).

Discussion

Malignant transformation of cells need changes of gene phenotype and the angiogenesis. VEGF is considered to be the most important angiogenic fator, and a large number of studies confirm that overexpression of VEGF can promote tumor growth, invasion and metastasis (Chang et al., 2009).

Stefanoup et al. (2003) reported that the expression of VEGF was 77.3% in NSCLC. In this study, expression rate of VEGF protein was 79.4% in NSCLC, while in normal lung tissues, expression rate of VEGF protein was 16.0%. There was significant difference between the two groups, (P <0.01), this suggests that the positive expression of VEGF cause the development of NSCLC and it has an important relationship to NSCLC. The current study suggests that VEGF can produce a marked effect by binding to receptors on endothelial cell membrane. After VEGF bind Flt-1 and Flk-1, it can directly stimulate the differentiation, proliferation and migration of pulmonary vascular endothelial cell, increase vascular permeability, change extracellular matrix, induce angiogenesis, and promote the growth of NSCLC (Wellmann et al., 2001).

In this study, the statistical analysis showed that the expression of VEGF was related to differentiation degree, lymph node metastasis and clinical Staging (P<0.01), while independent of other clinicopathologic parameters in NSCLC (P>0.05).The positive expression of VEGF was higher in poorly differentiated cancer, lymph node metastasis and clinical stage of III + IV. Thus, overexpression of VEGF has an important relationship to invasion.

The reason why VEGF is highly expressed in NSCLC is that it is regulated by angiogenin and some transcription factors. Among them, EGFR and MMP-9 are more important.EGFR is one of the tyrosine kinase receptor typeIgene family, mainly involved in signal transduction of cell. Once activated, it can induce cell differentiation, proliferation, invasion and angiogenesis (Berghmans et al., 2005, Krause and Van, 2005).

Jeon et al. (2006) investigate and discover that the positive rate of EGFR was 69.7% in NSCLC. In this study, the experiment results showed that the expression rate of EGFR was 75.0% in NSCLC, and was 12.0% in normal lung tissue. The expression of EGFR was significantly higher in NSCLC than in normal lung tissue, suggesting that EGFR may be involved in the occurrence of NSCLC. In this research, the statistical analysis showed that protein expression of EGFR was related to differentiation degree, lymph node metastasis and clinical staging in NSCLC (P<0.01), while independent of other clinicopathologic parameters (P>0.05) The results further suggest that the expression of EGFR may be involved in the development of NSCLC, and closely related to the degree of malignancy, invasion and metastasis. Possible molecular mechanism why EGFR promote development of NSCLC is that activation EGFR hasten the expression of VEGF and MMP-9. Thus, EGFR can be used as an important indicator to evaluate the degree of malignant, invasion in NSCLC.

MMP-9 is an enzyme with the largest molecular weight of MMPs, it secretes as zymogen. After activated, MMP-9 can hydrolyze basement membrane of cells and extracellular matrix so that the tumor cells can infiltrate connective tissue, small blood vessels, lympgatic vessels, and finally, the tumor cells occurrence metabasis. Recent studies show that MMP-9 can also promote angiogenesis (Hofmann et al., 2000, Sinnamon et al., 2008).

This experiment showed that the expression rate of MMP-9 was 68.4% in NSCLC, while the expression of MMP-9 was 8.0% in normal lung tissue. The expression of MMP-9 was significantly higher in NSCLC than in normal lung tissue, it also suggested that MMP-9 might be involved in the occurrence of NSCLC. In addition, we also found that protein expression of MMP-9 in poor differentiation, clinical stage of III and IVand lymph node metastasis was significantly higher than in clinical stage of I and II and had not lymph node metastasis of NSCLC. It suggested that MMP-9 was closely related to the invasion and metastasis.

In this study, Spearman rank correlation analysis showed that there was a obvious positive correlation of protein expression between VEGF and EGFR, that was, the higher the expression level of VEGF, the higher the expression of EGFR would be also. Induced activation of EGFR may evoke excessive proliferation of the cell so that the cell's demand for oxygen increases,

thereby contributes to upregulation of VEGF (Byers and Heymach, 2007). In NSCLC, the expression of VEGF, MMP-9 and the expression of EGFR, MMP-9 also positively correlated, suggesting that, VEGF can promote angiogenesis in process of invasive growth and metastasis. At the same time, MMP-9 may increase the degradation of extracellular matrix, simultaneous, the decline of homotypic adhesion and degradation of extracellular matrix can be a vicious cycle and promote each other. This may indicate that a synergistic effect among the three working together promote the occurrence of NSCLC, angiogenesis, invasion and metastasis.

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The authors declare that there is no conflict of interest with this work.

References

Berghmans T, Meert AP, Martin B, et al (2005). Prognostic role of epidermal growth factor receptor in stage III nonsmall cell lung cancer. *Eur Respir J*, **25**, 329-35.

Byers LA, Heymach JV (2007). Dual targeting of the vascular endothelial growth factor and epidermal growth factor receptor pathways: rationale and clinical applications for non-small cell lung cancer. *Clin Lung Cancer*, **8**, 79-85.

Chang SH, Kanasaki K, Gocheva V, et al (2009).VEGF-A induces angiogenesis by perturbing the cathepsin-cysteine protease inhibitor balance in venules, causing basement membrane degradation and mother vessel formation. *Cancer Res*, **69**, 4537-44.

Di Cristofano A, Pesce B, Cordon-Cardo C, et al (1998). PTEN is essential for embryonic development and tumor suppression. *Nat Genet*, **19**, 348-55.

Hofmann UB, Westphal JR, Van Muijen, et al (2000). Matrix metalloproteinases in human melanoma. *J Invest Dermatol*, **115**, 337-44.

Jemal A, Siegel R, Ward E, et al (2008). Cancer statistics 2008. *CA Cancer J Clin*, **58**, 71-96.

Jeon YK, Sung SW, Chung JH, et al (2006). Clinicopathologic features and prognostic implications of epidermal growth factor receptor (EGFR) gene copy number and protein expression in non-small cell lung cancer. *Lung Cancer*, **54**, 387-98.

Kojima H, Shijubo N, Yamada G, et al (2005). Clinical significance of vascular endothelial growth factor2C and vascular endothelial growth factor receptor 3 in patients with T1 lung adenocarcinoma. *Cancer*, **104**, 1668-77.

Krause DS, Van Etten RA (2005). Tyrosine kinases as targets for cancer therapy. *N Engl J Med*, **353**, 172-87.

Rami-Porta R, Crowley JJ, Goldstraw P (2009). The revised TNM staging system for lung cancer. *Ann Thorac Cardiovasc Surg*, **15**, 4-9.

Safranek J, Pesta M, Holubec L, et al (2009). Expression of MMP-7, MMP-9, TMP-1 and TMP-2 mRNA in lung tissue of patients with non-small cell lung cancer(NSCLC) and benign pulmonary disease. *Anticancer Res*, **29**, 2513-7.

Sinnamon MJ, Carter KJ, Fingleton B, et al (2008). Matrix metalloproteinase-9 contributes to intestinal tumourigenesis in the adenomatous polyposis coli multiple intestinal neoplasia mouse. *Int J Exp Pathol*, **89**, 466-75.

Stefanoup D, Goussia AC, Arkoumani E, et al (2003). Expression of vascular endothelial growth factor and the adhesion molecule E-cadherin in non small cell lung cancer. *Anti Cancer Res*, **23**, 4715-20.

Wellmann S, Taube T, Paal K, et al (2001). Specific reverse transcription-PCR quantification of vascular endothelial growth factor (VEGF) splice variants by LightCycler technology. *Clin Chem*, **47**, 654-60.