Relationship between Lymphatic Metastasis and Expression of c-met Proto-oncogene and Microvessel Density (MVD) in Lung Cancer

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Abstract **objective** To study the expression of c-met proto-oncogene and microvessel density MVD) in lung cancer, and their roles in lymphatic metastasis. **Methods** Immunohistochemical technique was used to detect the expression of c-met and FⅧ-RAg protein and in situ hybridization was employed in the expression of c-met proto-oncogene mRNA in 62 clinical specimens. **Results** Expression of c-met protein and mRNA were correlated significantly with tumor differentiation, size, stage and lymph node metastasis ($P<0.05$). Also, there was a significant correlation between the expression of c-met and nodal status of lymph node metastasis ($P<0.05$). C-met expressions in metastatic lymph nodes were significantly increased compared with those in the primary site ($P<0.01$). There were significant correlations between MVD and tumor stage, lymph node metastasis ($P<0.05$). MVD was significantly higher in c-met positive tissues than those in c-met negative tissues ($P<0.01$). **Conclusion** C-met enhances the invasive ability of the cancer cells and induces angiogenesis, may play an important role in invasion and metastasis of lung cancer.

Key Words c-met proto-oncogene; Microvessel density; Lung cancer; Lymph node metastasis

The human c-met oncogene encodes a transmembrane tyrosine kinase with structural and functional features of a growth-factor receptor. The ligand for the c-met receptor is hepatocyte growth factor (HGF), also known as scatter factor (SF). HGF/SF-c-met signaling has been shown to trigger a variety of cellular responses that vary based upon the cellular context. It is believed that the HGF/SF-c-met signaling system plays an important role in cell proliferation and differentiation, angiogenesis, tumor invasion and metastasis[1].

To gain insight into the biological function in tumor invasion and metastasis of c-met receptor, we have detected the expression of c-met protein and mRNA in 62 lung cancer clinical specimens. We also discussed the relationship between c-met expression and microvessel density (MVD) in lung cancer.

MATERIALS AND METHODS

Tumor samples

Sixty-two fresh lung cancer tissues and 29 metastatic lymph nodes were collected from Tongji Hospital of Tongji medical college of Huazhong University of Science and Technology in 2001. All patients were not subjected to chemo- and radiotherapy. Tumor grade and pathological stage were assessed according to the WHO standard of lung cancer. The final diagnosis of the carcinomas examined was 29 squamous cell carcinoma and 33 adenocarcinamas. Among all samples, there were 14 highly differentiated, 25 moderately differentiated and 23 low differentiated lung cancer. The TNM staging system of tumor tissues was as follows: 32 in Ⅰ stage, 16 in Ⅱ stage, 11 in Ⅲ stage and 3 in Ⅳ stage. Several resected specimens were fixed in 10% formalin for HE and immunohistochemical analysis, others were fixed in 4% paraformaldehyde for in situ hybridization. Both were embedded in paraffin. Serial 3μ m sections were applied to polylysine-coated slides and dried at 50℃ for at least 2 hours.

Immunohistochemical Analysis

Staining was made by S-P method following the illustration. Affinity purified polyclonal antibodies against human met (1:80) and factor Ⅷ-related antigen (FⅧ-RAg) (1:50) was obtained from Santa Cruz biotechnology(C-28) and Zhongshan biotechnology of Beijing (ZA-0111). Negative controls was performed using phosphate buffered saline (PBS) buffer instead of the primary antibody, while positive controls used the positive section.

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In Situ Hybridization

C-met ISH detection Kit was purchased from Wuhan Bosde Biotechnology Ltd. China. The experiment was carried out following the illustration. Negative controls was performed using phosphate buffered saline (PBS) buffer instead of the probe, while positive controls used the positive section.

Determination of Results

C-met protein localized on the cell membrane, some cytoplasm also stained in Immunohistochemistry. In situ hybridization the cytoplasm of tumor cells showed diffuse staining. Based on the percentage of cells showing definite staining over at least 1000 tumor cells counted in randomly selected high-power(×400) fields, the samples were classified into two groups negative tumors (<25% immunoreactive tumor cells) and positive tumors (>25% immunoreactive tumor cells).

The standard of microvessel density(MVD): Count the number of microvessels under selected 5 different and most FⅧ-RAg staining fields(×200), then calculate the mean of these 5 numbers as MVD($\bar{X}\pm S$)[2].

Statistical Analysis

All statistical analysis were performed using the computer software SPSS. Difference was considered significant when the P value was less than 0.05.

RASULTS

Relationship between c-met expression and the clinical feature of lung cancer

When comparing carcinoma areas with adjacent normal tissues, a stronger reactivity for the c-met receptor was detected in carcinomas. The relationship between tumor's biological characters and the expression of c-met protein and mRNA were summarized in table 1. Among 62 lung cancer, the positive rate of c-met protein expression was 75.8% and c-met mRNA was 74.2%. It was observed that c-met expression were elevated gradually with the higher TNM staging, lower differentiated and lymph node metastatic tumors. (Fig., Fig. 2).

Correlation with lymphatic metastasis of lung cancer

Twenty six out of 29 (26/29, 89.7%) matastatic lymph nodes displayed much higher c-met expression significantly ($P<0.05$) than those of the primary site (47/62, 75.8%). Using HPIAS-1000 analysis system, matastatic lymph nodes (0.2186±0.0283) were significantly stronger staining than primary site (0.1362±0.0266)($P<0.01$). Meanwhile, c-met expressions were increased with tumor's Nodal Status ($P<0.05$). The positive rate was higher in more lymph node metastasis, but the difference was not statistically significant (Table 2, Fig 3).

Table 1 Relationship between the expression of c-met and the clinical feature of lung cancer

Clinical feature	n	c-met protein		X^2	*P*	c-met mRNA		X^2	*P*
		-	+(%)			-	+(%)		
Histology									
Squamous cell	29	9	20(69.0%)	1.3903	0.2384	10	19(65.5%)	2.1422	0.1433
Adenocarcinoma	33	6	27(81.1%)			6	27(81.8%)		
Differentiation									
High	14	6	8(57.1%)	8.4289	0.0148*	7	7(50.0%)	10.3122	0.0058*
Moderate	25	8	17(68%)			8	17(68%)		
low	23	1	22(95.7%)			1	22(95.7%)		
Tumor size									
<4cm	23	9	14(60.9%)	4.4480	0.0349*	10	13(56.5%)	5.9638	0.0146*
≥ 4cm	39	6	33(84.6%)			6	33(84.6%)		
Lymph metastasis									
Negative	33	12	21(63.6%)	5.6976	0.0170*	12	21(63.6%)	4.1069	0.0427*
Positive	29	3	26(89.7%)			4	25(86.2%)		
TNM staging									
Ⅰ	32	11	21(65.6%)	6.2824	0.0432*	11	21(65.6%)	6.3443	0.0419*
Ⅱ	16	4	12(75%)			5	11(68.8%)		
Ⅲ+Ⅳ	14	0	14(100%)			0	14(100%)		

*$P<0.05$

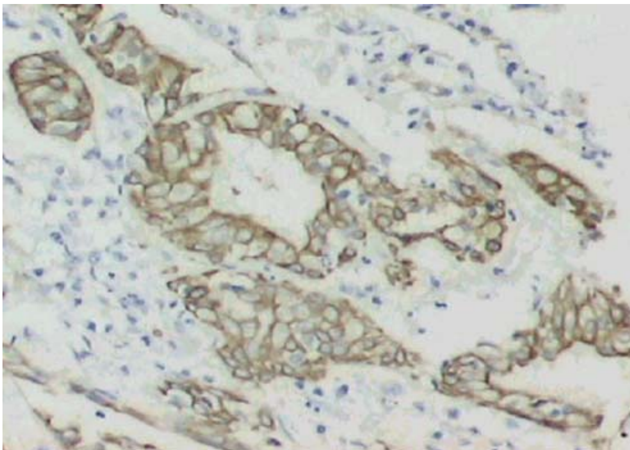

Fig.1 Expression of c-met in lung cancer, the positive brown staining granules were seen in cell membrane of cancer cells, and some cytoplasm cvere also stained (SP ×200)

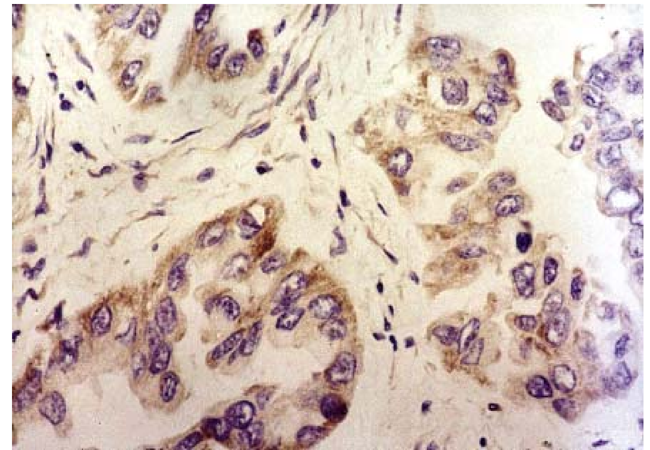

Fig.2 Expression of c-met mRNA in lung cancer, the positive brown staining granules were seen in cytoplasm of cancer cells by in situ hybridization.(ISH×200)

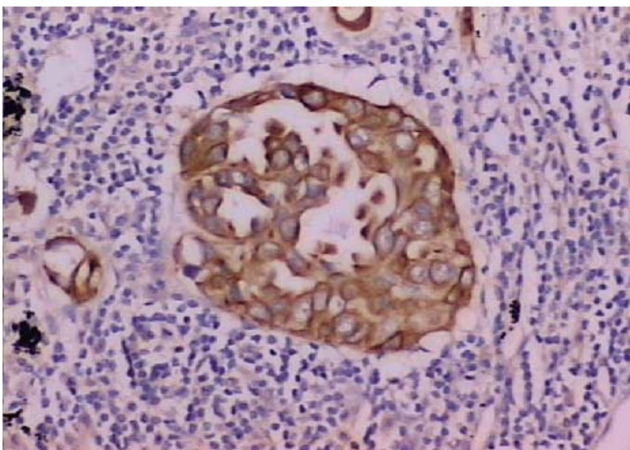

Fig.3 Expression of c-met in metastatic lymph nodes of lung cancer (SP×200)

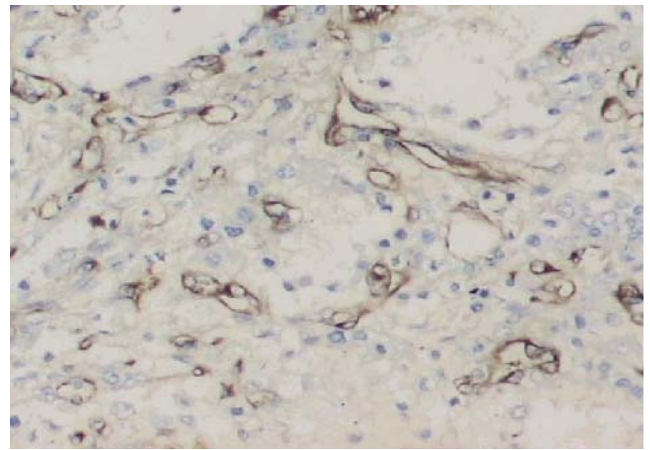

Fig.4 Microvessels were stained by FⅧ-RAg antibody in lung cancer (SP×200)

Table 2 Expression of c-met protein in metastatic lymph nodes of lung cnacer

	n	c-met		Positive rate (%)	X^2	p
		-	+			
Lymph node metastasis						
Negative	33	12	21	63.6%	5.6976	0.0170*
Positive	29	3	26	89.7%		
Number of metastatic lymph node						
<4	20	3	17	85%	1.5058	0.2198
≥ 4	9	0	9	100%		
Nodal Status						
N_0	33	12	21	63.6%	7.0724	0.0291*
N_1	16	3	13	81.3%		
≥ N_2	13	0	13	100%		

* $P<0.05$

Relationship between MVD and lung cancer metastasis

Microvessels were detected diffusely in tumor and tumor stroma (Fig 4). MVD was significantly related to TNM staging and lymph node metastasis ($P<0.05$).

Table 3 Relationship between MVD and lung cancer metastasis

		n	MVD($\bar{x}\pm s$)	P
Differentiation				
	High	14	45.19±6.58	0.995
	Moderate	25	45.63±8.68	
	Low	23	45.10±8.10	
Tumor size				
	< 4cm	23	43.85±7.81	0.131
	≥ 4cm	39	47.13±8.28	
TNM staging				
	I	32	40.77±14.78	0.006*
	II	16	44.58±6.13	
	III+IV	14	52.47±7.89	
Lynph node metastasis				
	Negative	33	44.70±6.07	0.022*
	Positive	29	47.41±2.27	
Number of metastatic lymph node				
	< 4	20	43.33±7.29	0.053
	≥ 4	9	51.80±10.72	

* $P<0.05$

Table 4 Correlation between MVD and c-met protein expression

c-met	n	MVD(±s)	P
-	15	45.64±8.40	0.001*
+	47	55.00±10.22	

*$P<0.01$

However, no significant association was found between MVD and several clinicophathological characteristics, including tumor size, differentiation and number of metastatic lymph nodes (Table 3).

Correlation between MVD and c-met protein expression

Table 4 showed that MVD was significantly higher in the group of c-met protein positive tumor than those in non-expressing c-met protein group (P<0.05), which indicated that c-met oncogene induces angiogenesis in lung cancer.

DISCUSSION

The c-met proto-oncogene, initially identified as a transforming gene from a chemically treated human osteogenic sarcoma cell line, encodes a transmembrane glycoprotein with the characteristics of a tyrosine kinase receptor. The protein is a 190kDa two-disulfide-linked chains (p190c-Met), composed of the extracellular domain, the transmembrane domain and the intracellular domain. The COOH-terminal portion of the β chain contains the tyrosine kinase domain and phosphorylation sites involved in the regulation of its activity and in signal transduction[3]. The c-met protein is expressed in many kinds of cell, mainly in epithelial cells of a variety of organs [4]. Its ligand, hepatocyte growth factor (HGF), is secreted by mesenchymal cells[5]. Being a potent stimulator of cell scattering, HGF was named scatter factor (SF). When binding to its ligand, c-met receptor triggers a cascade of tyrosine phosphorylation and elicits mitogenic, motogenic, and morphogenic actions on a wide variety of target cells [6]. This wide array of cellular responses allows HGF/SF-c-met signaling to be involved in a number of biological processes that involve interaction between mesenchymal and epithelial cells. Such paracrine signaling likely plays a role in tumor growth, invasion, metastasis, angiogenesis and poliferation[7]. In our experiment, the positive rate of c-met protein expression was 75.8% and c-met mRNA was 74.2% . Significant relationship were found between c-met expression and several clinicopathological characteristics, including differentiation, TNM staging, lymph node metastasis and nodal status. It was observed that c-met expression was elevated gradually with the higher TNM staging, lower differentiated, lymph node metastatic and higher nodal status tumors. There was no significant difference between c-met expression and histology, tumor size, and number of metastatic lymph

nodes, but the positive rate was higher in adenocarcinoma, bigger tumor and more lymph node metastasis. Metastatic lymph nodes displayed significantly higher c-met expression than those in the primary site. These suggested that c-met oncogene likely enhance tumor cell's invasive ability and play an important role in development of lung cancer.

The induction of angiogenesis (formation of new blood vessels) may be an important mechanism that permits tumor cell proliferation and eventually metastasis. Tumor-derived angiogenic factors lead to ingrowth of new capillaries with fragmented basement membranes, allowing tumor cells to more rapidly enter the circulation[8]. HGF/SF acts as a potent angiogenic molecule by directly acting on vascular endothelial cells. HGF/SF can also promote the expression of other angiogenesis factors by tumor cells, such as IL-8 and vascular endothelial growth factor (VEGF)[9]. In addition, HGF/SF stimulated pathway is important in the up-regulation of expression of the serine protease urokinase(uPA) and its receptor (uPAR), which degrade several extracellular matrix (ECM) and basement membrane (BM) components[10]. Thus HGF/SF induces angiogenesis by promoting vascular endothelial cells migration, proliferation, protease production, invasion, and organization into capillary-like tubes[11]. MVD is a good quantitative index to assess angiogenesis in tumor[2]. In our present study, MVD was significantly related to TNM staging and lymph node metastasis. MVD and c-met expression were significantly association, MVD was much higher in the group of c-met protein positive tumors than those in non-expressing c-met protein group.

The results of our study indicated that c-met oncogene can enhance invasive ability of tumor cells and induce angiogenesis, directly involved in lung cancer invasion and metastasis. Further study on its mechanism and inner molecular details would be worthwhile.

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