RESEARCH COMMUNICATION

Expression and Clinical Significance of STAT3, P-STAT3, and VEGF-C in Small Cell Lung Cancer

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Abstract

Objective: To determine STAT3, P-STAT3, and VEGF-C expression levels in small cell lung cancers (SCLCs), and discuss their role and clinical significance in SCLC development. Method: Immunohistochemical methods were applied to 128 cases of SCLC and 40 cases of adjacent normal tissue. Results: The expression levels of STAT3, P-STAT3, and VEGF-C were higher in SCLC than in normal tissue ($P < 0.05$). Pairwise comparisons showed positive correlations with lymph node metastasis, clinical stage, and tumor size ($P < 0.05$). The expression levels were also related with the overall survival rates. Conclusion: STAT3 and VEGF-C play important roles in the development of SCLC, and might be expected to become new targets for SCLC treatment.

Keywords: STAT3 - P-STAT3 - VEGF-C - SCLC - survival

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Introduction

Lung cancer currently has the highest incidence and fatality rate of malignant tumors worldwide. Small cell lung cancer (SCLC) accounts for 15% to 20% of lung cancers, and is divided into wide- and limited-range survival rates (Felip et al., 2005). The U.S. SEER epidemiological survey database in the past 30 years shows a downward trend in the annual incidence of SCLC (Govindan et al., 2006). However, most patients are diagnosed at the late stage due to the short and rapid cell doubling time, as well as early blood transfer occurrence. Thus, SCLC therapy is difficult. The long-term survival of SCLC is low; the limited and extensive five-year survival rates are only 10% and 2%. Therefore, a complete understanding of the molecular mechanisms of lung cancer would contribute to the development of new treatments.

Signal transduction and activator of transcription factor (STAT) is a family of proteins that exists in the cytoplasm. These proteins can be transferred to the nucleus and bind to DNA after activation. The members of the STAT family include STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Fu et al., 1992). STAT3 is the most closely associated with tumors. The gene encoding STAT3, which is located on chromosome 12, consists of 750 to 850 amino acids and has a molecular weight of 84–113 kDa. STAT3 can be activated in three ways, namely, via the JAK-Stat way, Ras-MAPK approach, and size-independent enzymatic activation. Among them, the classic one is the Stat3 JAK-Stat way of signaling and transcription. In a number of cytokines and growth factor stimulators, STAT3 activates tyrosine or serine kinase phosphorylates STAT3 (P-STAT3). A dimer then forms and transfers to the nucleus of the target gene promoter binding region. Consequently, the inhibition of apoptosis, gene expression of Bcl-XL, cyclinD1/D2 expression, vascular endothelial growth factor expression, and blood vessel formation are directly or indirectly increased (Xu et al., 2005). STAT3 activation is also involved in cell proliferation, apoptosis, differentiation, and other physiological activities (Zhang et al., 2010). At present, P-STAT3 is abnormally expressed in leukemia, multiple myeloma, breast cancer, ovarian cancer, prostate cancer, bladder cancer, head and neck squamous cell carcinoma, malignant melanoma, lung cancer, glioma, lymphoma, colon cancer, and kidney cancer.

Tumor growth and development is a complex multi-step process in which angiogenesis is one of the essential steps and plays a vital role in tumor invasion and metastasis. Vascular endothelial growth factor is considered to be the main stimulating factor of tumor-associated angiogenesis. In a melanoma mouse model, vascular endothelial growth factor is a direct target of STAT3, thus confirming the role of STAT3 in angiogenesis for the first time (Niu et al., 2002). The in vitro and in vivo regulation of STAT3 activities in human melanoma apparently influences the bFGF, VEGF, and MMP-2 expression of angiogenic factors, thereby affecting the angiogenesis and brain metastases of melanoma cells (Xie et al., 2006). Niu et al. (2002) found that STAT3 can directly activate the expression of VEGF and stimulate tumor angiogenesis. VEGF expression decreases STAT3 gene fragment by mutation or blocking. These results suggest that STAT3 is a common molecular target for the

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multi-factor blocking of tumor cell-induced angiogenesis.

Materials and Methods

Clinical data

A total of 128 thoracic surgical SCLC specimens from January 2001 to December 2010 from 66 male and 62 female patients were used. There were 70 patients who had a history of smoking. Regarding the postoperative TNM stage, 40 were stage I, 37 were stage II, 28 were stage III, and 23 were stage IV. There were 69 cases of lymph node metastasis, and 59 without lymph node metastasis. All patients were followed up, and the survival rate from the surgery to death date or December 31, 2010 was recorded after a median follow-up time of 15.4 months. No preoperative radiotherapy or chemotherapy treatment was conducted. The largest cut surface of the tumor location was serially sectioned (4 μm). Lung cancer around the organ was observed more than 5 cm from the cancer tissue. All patients signed informed consents. This study was approved by the Ethics Committee of Harbin Medical University.

Immunohistochemistry

Mouse anti-human STAT3 monoclonal antibody, mouse anti-human P-STAT3 (B-7) monoclonal antibody, and mouse anti-human VEGF-C monoclonal antibody were obtained from Wuhan Boster Company. An immunohistochemical SP kit and a DAB coloration kit were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. The SP of STAT3, P-STAT3, and VEGF-C protein was detected by immunohistochemical staining. After dewaxing and hydrating the paraffin sections, they were washed with PBS (pH 7.4) and water thrice for 3 min each time, and then with 3% H2O2. The sections were incubated for 10 min to eliminate endogenous peroxidase activity. The sections were washed again with PBS thrice for 3 min each time, and an appropriate proportion of an anti-wet box was added for dilution. The sections were subsequently incubated for 60 min at room temperature, washed with PBS thrice for 5 min each time, and added with two drops of anti-horseradish peroxidase poly body fluid. After incubation at room temperature for 10 min to 30 min, PBS washing thrice for 3 min each time was performed. DAB was added and the color was observed for 5 min to 10 min under a microscope. After adding distilled water, the sections were counterstained with hematoxylin. Alcohol differentiation using 0.1% hydrochloric acid and PBS washing for color reversion were performed. The DAB color slices were subjected to alcohol dehydration and treated with xylene, sex gum, the seal the tablet Fengpian, number. A negative control slide that did not contain the primary antibody was also prepared.

Results

STAT3 protein positive staining in the cytoplasm showed that some nuclei appeared as brown particles. The P-STAT3 positive protein was localized in the nucleus, whereas VEGF-C positive staining was located in the cytoplasm and membrane. The cell staining intensity was in the proportion of points, and two points determined the final outcome. The staining intensity was classified as follows: did not stain, 0 point; mild coloring, 1 point; moderate coloring, 2 points; strong coloring, 3 points. All slices were observed under five random high-power fields and averaged. The number of positive cells were as follows: no coloring or 0 point, <25%; 1 point, 25% to 50% for 2 min and 50% to 75% for 3 min; 4 points, >75%; 2 points together: -1 to 2 for the weak positive, 3 to 4 +, 5 + +; divided into 7 + + +; semi-quantitative judgment.

Data analysis

SPSS 18.0 software was used for all statistical analyses. The c2 test was used for STAT3, P-STAT3, and VEGF-C expression and clinical stage. The expression levels of STAT3, P-STAT3, and VEGF-C in lung cancer tissues were determined using the Spearman correlation coefficient analysis method. The Kaplan-Meier method was employed to draw survival curves and for log-rank test verification. Univariate regression analysis using the Cox proportional hazards model and multivariate regression analysis were applied to determine the independent factors related to prognosis. For all tests, $P < 0.05$ was considered statistically significant.

STAT3, P-STAT3, and VEGF-C expression

Figure 1 shows a diagram of STAT3, P-STAT3, and VEGF-C immunohistochemical staining. Positive cells appeared as brown particles. STAT3 protein was mainly located in the cytoplasm, and some nuclei appeared as

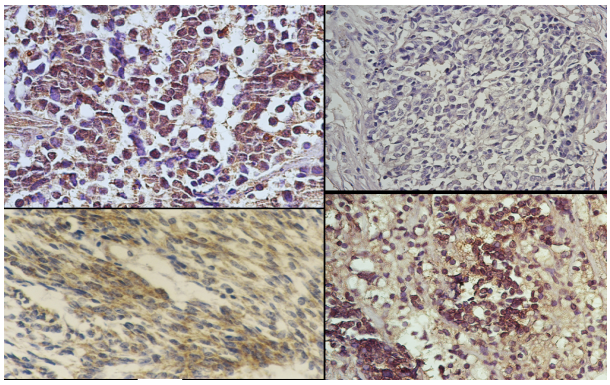

Figure 1. Of STAT3, P-STAT3 and VEGF-C in Small Cell Lung Cancer and Adjacent Normal Tissues by Immunohistochemical Staining (SP×400). (A) STAT3 protein positive stain in the cytoplasm, some nuclei appears brown particles. (B) Positive for P-STAT3 protein in the nucleus. (C) VEGF-C positive staining in the cytoplasm and membrane. (D) Normal lung tissue

Table 1. Correlations Between STAT3, P-STAT3 and VEGF-C Expression in SCLC

	P-STAT3				VEGF-C			
	-	+	r	P	-	+	r	P
STAT3								
-	35	22	0.1764	0.0459	35	22	0.2609	0.0032
+	31	40			25	46		
VEGFC								
-	40	20	0.2839	0.0013				
+	26	42						

Table 2. Correlation Between Expression of STAT3, P-STAT3 and VEGF-C and Clinicopathological Features

Features		All patients (n=128)	STAT3 - (n=57)	STAT3 + (n=71)	P	P-STAT3 - (n=66)	P-STAT3 + (n=62)	P	VEGFC - (n=60)	VEGFC + (n=68)	P
Year	≥60	64	27	37	0.5937	34	30	0.7235	28	36	0.4786
	<60	64	30	34		32	32		32	32	
Gender	Male	66	32	34	0.3531	36	30	0.486	28	38	0.2978
	Female	62	25	37		30	32		32	30	
Smoking	Yes	70	33	37	0.5137	36	34	0.9734	32	38	0.7725
	No	58	24	34		30	28		28	30	
Lymph node status	Yes	69	18	51	0.001	28	41	0.0072	18	51	<0.0001
	No	59	39	20		38	21		42	17	
Tumor size	≥3cm	70	20	50	<0.0001	26	44	0.0027	18	52	<0.0001
	<3cm	58	37	21		37	21		42	16	
Stage	I		32	8	<0.0001	33	7	<0.0001	33	7	<0.0001
	II	40	16	21		18	19		17	20	
	III	37	5	20		8	17		6	19	
	IV	28	4	22		7	19		4	22	

Table 4. Univariate and Multivariate Analyses of Prognostic Factors for Survival of the SCLC Patients

Variables	Univariate analysis HR	95%CI	P-value	Univariate analysis HR	95%CI	P-value
Gender(Male vs Female)	1.181	0.802 - 1.739	0.3998	1.285	0.860-1.921	0.2206
Age (<60 vs. ≥60 years)	0.88	0.598-1.294	0.5153	1.085	0.718-1.639	0.6988
Smoking (Yes vs. no)	1.162	0.790-1.710	0.4468	1.381	0.911-2.095	0.1286
Lymph node status (Yes vs. no)	3.836	2.472-5.952	<0.0001	1.858	1.113-3.102	0.0178
Tumor size(<3cm vs.≥3cm)	3.736	2.383-5.857	<0.0001	1.851	1.136-3.015	0.0134
Stage(I II vs III IV)	4.895	3.551-6.746	<0.0001	3.662	2.542-5.277	<0.0001
STAT3(- vs +)	5.008	3.137-7.995	<0.001	2.368	1.367-4.102	0.0021
P_STAT3(- vs +)	3.013	1.896-4.790	<0.0001	1.106	0.680-1.798	0.6845
VEGFC(- vs +)	4.79	2.607-2.263	<0.0001	1.956	1.169-3.272	0.0106

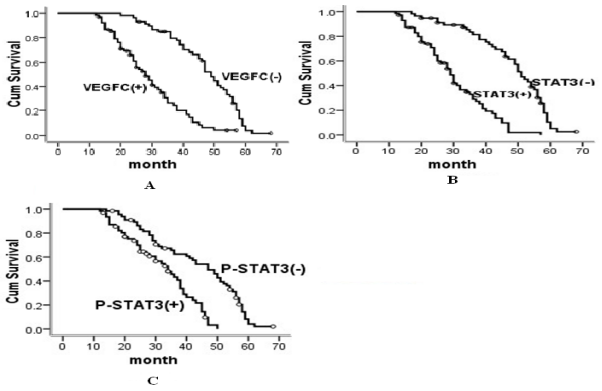

Figure 2. STAT3, P-STAT3, of VEGF-C Expression Levels and Overall Survival of Patients with SCLC. (A) STAT3-positive patients with lung cancer overall survival time was significantly lower in patients with STAT3-negative tumors, P <0.05. (B) P-STAT3-positive patients with lung cancer overall survival time was significantly reduced STAT3 negative tumors, P <0.05. (C) VEGF-C positive patients with lung cancer overall survival time was significantly lower in patients with STAT3-negative tumors, P <0.05

brown particles (Figure 1a). P-STAT3-positive protein was observed in the nucleus (Figure 1b). VEGF-C positive staining was observed in the cytoplasm and membrane (Figure 1c). The immunohistochemical results showed that STAT3, P-STAT3, and VEGF-C expression were higher in lung cancer tissue than in adjacent normal tissue (P < 0.05). The expression rates were 71 (55.4%), 62 (48.4%), and 68 (53.1%). P-STAT3 and STAT3 (R = 0.176, P = 0.045), P-STAT3 and VEGF-C (R = 0.283, P = 0.001),

Table 3. STAT3, P-STAT3, of VEGF-C Expression Levels and Survival of Overall Patients with SCLC

	one year survival rate %	three year survival rate %	five year survival rate %
STAT3(-)	100	81.4±5.3	10.2±4.7
STAT3(+)	98.6±1.4	33.0±6.0	0
P- STAT3(-)	100	62.3±6.1	4.1±2.8
P- STAT3(+)	98.4±1.6	43.2±7.0	0
VEGF-C(-)	100	79.4±5.3	4.0±2.8
VEGF-C(+)	98.5±1.5	24.2±6.0	0

and STAT3 and VEGF-C (R = 0.260, p = 0.003) between any two comparisons were positively correlated (Table 1).

Relationship between protein expression and clinical features

The relationships of STAT3, P-STAT3, and VEGF-C expression with the clinical features of SCLC are shown in Table 2. The STAT3, P-STAT3, and VEGF-C expression levels were positively correlated with lymph node metastasis, clinical stage, and tumor size (P < 0.05). The expression levels were also correlated with the patient age and gender, but not to the smoking status.

STAT3, P-STAT3, and VEGF-C expression levels and overall survival

The overall survival times of STAT3-, P-STAT3-, and VEGF-C-positive patients were significantly lower than those of STAT3-, P-STAT3-, and VEGF-C-negative patients (P < 0.05) (Figure 2). The one-, three, and five-

year survival rates of STAT3-, P-STAT3-, and VEGF-C-positive patients were significantly lower than those of patients negative for these three proteins (Table 3).

SCLC survival prognostic factors and multivariate analysis

Univariate analysis showed that tumor size, lymph node metastasis, clinical stage, and expression of STAT3, P-STAT3, and VEGF-C were positively correlated with the overall patient survival. We conducted a multivariate survival analysis to describe the statistics of the experimental results. Our results showed that tumor size, lymph node metastasis, tumor stage, STAT3 expression, and VEGF-C expression were independent predictors (Table 4).

Discussion

STAT3, which exists in a variety of human cancer cells via different cytokines, is involved in growth factor activation and regulation of the tumor malignant process (Bromberg, 2002; Yu and Jove, 2004; Al Zaid Siddiquee and Turkson, 2008). STAT3 either directly or indirectly regulates genes related to cell proliferation, metastasis, and survival time (Garcia et al., 2001; Barbieri et al., 2010). STAT3 is important in regulating the survival of human non-SCLC (NSCLC) (Song et al., 2003). In glioblastoma stem cells (GSCs), STAT3 induces apoptosis and significantly reduces the expression of BCL-2 and cyclin-D, indicating that STAT3 is an important target of human GSCs (Li et al., 2010). A previous study (Xu et al., 2003) demonstrated that STAT3 expression is significantly higher in NSCLC tissues than in the adjacent tissues. STAT3 expression was higher in poorly differentiated than in well-differentiated groups. STAT3 expression was higher in lymph node metastasis than without lymph node metastasis. These results indicate that STAT3 expression is highly associated with NSCLC, tissue differentiation, and lymph node metastasis. Huang et al. (2008) found that the transfection of STAT3-siRNA-1 and STAT3-siRNA-2 significantly decreases STAT3 expression in SCLC, indicating the relation of the proliferative activity of the STAT3-siRNA vector with SCLC. In the present study, STAT3 protein expression had great frequency (55.4%) in 128 cases of SCLC. STAT3 expression was also positively correlated with tumor stage, lymph node metastasis, and tumor size. Therefore, STAT3 may be a novel biomarker for determining the progression and prognosis of SCLC.

STAT3 is an activated form of P-STAT3. The activation of STAT3 factors include cytokines, growth factors, and even cancer genes, including epidermal growth factor, platelet-derived growth factor, IL-6 Src, and Ras (Liu et al., 2011). STAT3 regulates melanoma cell matrix metalloproteinase-2 (MMP-2) gene transcriptional activity (Xie et al., 2004, 2006), MMP-2 can degrade the basement membrane (Li et al., 2011), and P-STAT3 can upregulate vascular endothelial cell growth factor expression, resulting in tumor angiogenesis (Xu et al., 2005). Tumor angiogenesis has an important role in metastasis and invasion of many malignant tumors. STAT3 activity deletion can significantly inhibit the expression of MMP-2, tumor invasion, as well as brain and lung metastasis (Xie et al., 2004, 2006). Wang Meng et al. found a significant positive correlation between P-STAT3 and the lymph node status in NSCLC, suggesting that STAT3 activation in NSCLCs play an important role in tumor invasion and lymph node metastasis (Wang et al., 2011). In the present study, P-STAT3 was highly expressed in SCLC and not expressed in adjacent tissues. P-STAT3 expression was higher in lymph node metastasis than without lymph node metastasis. P-STAT3 expression was positively correlated with the clinical stage, but was not correlated with the gender and age. Therefore, P-STAT3 may play an important role in the progression and metastasis of SCLC.

VEGF-C is a new member of the vascular endothelial growth factor family. In 1996, Joukov et al. (1996) cloned for the first time a cDNA library from the human prostate cancer cell line PC-3 and isolated VEGF-C. VEGF-C expression is found to be correlated with lymph node metastasis in a variety of human cancer studies. VEGF-C increases the number of lymphatic vessels possibly by promoting lymphatic vessel formation, thus increasing the invasion of tumor cells and lymphatic contact area to promote transfer. Li-zhi Bai (2005) reported that VEGF-C protein expression in SCLC was significantly higher than that in normal lung tissue and adjacent tissues. Therefore, STAT3 and hypoxia-inducible factor 1α (HIF-1α) regulate VEGF. STAT3 is activated to promote HIF-1α expression under hypoxic conditions, resulting in the regulation of VEGF expression to promote the proliferation of tumor blood vessels (Ran et al., 2011). In the current work, 58.5% VEGF-C expression in SCLC samples was significantly correlation with lymph node metastasis, clinical stage, and tumor size. Hence, the overexpression of VEGF-C is a poor independent prognostic factor.

We also found the existence of a common expression of STAT3 and P-STAT3, STAT3 and VEGF-C, as well as VEGF-C and P-STAT3 Approximately 57.8% of SCLC samples with STAT3 expression were positively correlated with the clinical stage, lymph node metastasis, and tumor size. VEGF-C overexpression in SCLC is related with lymph node metastasis, tumor stage, and tumor size. The current research suggests that these proteins may play roles in SCLC progression and angiogenesis and that VEGF-C may promote metastasis via the STAT3 pathway. However, further research is needed to elucidate the detailed mechanisms.

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