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Original Article

Expression and clinical correlates of signal transducers and activators of transcription 4: a tissue microarray study of 365 lung cancers

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Abstract: Background: Signal transducer and activator of transcription 4 (STAT4) is a transcription factor belonging to the signaling pathways of several cytokines. STAT4 has been studied in several malignancies; however, its clinical value in lung cancer has not been fully clarified. Hence, the objective of this study was to explore the expression and clinicopathological significance of STAT4 in lung cancer tissues. Methods: STAT4 expression was determined by immunohistochemistry (IHC) in tissue microarrays with 365 lung cancer and 30 normal lung tissues. The correlations with clinical parameters of STAT4 expression was analyzed by Receiver operating characteristic (ROC) curve. The correlation between STAT4 expression and clinicopathological parameters was further investigated. Results: STAT4 expression in lung cancer, including both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), was all significantly higher than in non-cancerous normal lung (all $P<0.05$). ROC curve showed that the area under curve of STAT4 was 0.625 (95% CI 0.525~0.726, $P=0.022$) for lung cancer. In addition, STAT4 expression was associated to TNM stage ($P=0.003$), distal metastasis ($P=0.007$) and lymph node metastasis ($P<0.001$) in lung cancers. The consistent correlations between STAT4 and lymph node metastasis ($P<0.001$) and distal metastasis ($P=0.006$) were observed in NSCLC. Simultaneously, STAT4 expression was interrelated with clinical TNM stages ($P=0.001$) and lymph node metastasis ($P=0.001$) in SCLC. Conclusion: Our data demonstrated that STAT4 is upregulated in lung cancer. Higher STAT4 expression is associated with advanced stage and metastases, which indicates a potential tumorigenic role of STAT4 in the pathogenesis of lung cancer.

Keywords: STAT4, lung cancer, metastasis, tissue microarray immunohistochemistry

Introduction

Lung cancer possesses the highest morbidity among all malignant tumors, with an estimated incidence of more than 1.6 million patients per year, accounting for 13% of all new cancer diagnoses [1]. Among all lung cancers, 75-85% are non-small cell lung cancer (NSCLCs). The overall 5-year survival ratio of NSCLC remains at a low level less than 20%, mainly due to the advanced disease occurred in more than 70% of NSCLC patients at the moment of their diagnoses. In order to identify operative molecular markers for lung cancer diagnosis and prognosis, great efforts have been put into this research area, however, no specific markers have been discovered to date [2]. Hence, exploration for efficient molecular biomarkers remains still an essential issue to be settled.

Signal transducer and activator of transcription (STAT) can promote the transcription of target gene, including c-myc, Bcl-x_L, and cytokine signaling in inflammatory diseases, in nuclear promoter sequence. STAT4, a member of the STAT family, expresses in lymphocytes, macrophages, and dendritic cells, encoding a transcription factor that resides in the cytosol [3]. Recent studies have revealed that STAT4 is involved in a certain classes of cancers, such as gastric cancer [4], colorectal cancer (CRC) [5], lymphoma [6], breast cancer [7], classic Kaposi sarcoma [8] and hepatocellular carcinoma (HCC) [9, 10]. The expression of STAT4

Table 1. Characteristics of patients in the current study

Patients	Number of patients
Gender	
Male	275
Female	90
Age (years)	
<60	196
≥60	169
Histology	
SCLC	26
NSCLC	339
Adenocarcinoma	127
Acinar adenocarcinoma	83
Papillary adenocarcinoma	19
Broncholoalveolar cell carcinoma	18
Mucinous carcinoma	7
Squamous cell carcinoma	175
Adenosquamous carcinoma	28
Undifferentiated carcinoma	8
Large cell carcinoma	1
TNM	
I-II	299
III-IV	63
Lymph node metastasis	
Yes	128
No	234
Tumor diameter (cm)	
≤7	48
>7	314
Distal metastasis	
Absent	346
Present	0

protein was found to be statistically significant correlated with the tumor stage, distal metastasis and depth of invasion in HCC and CRC patients, which indicated STAT4 may be a promising biomarker in the diagnosis and prognosis in HCC and CRC patients. However, STAT4 may play different roles in distinct types of malignancies. For instance, STAT4 was upregulated in gastric cancer and colorectal cancer, functioning as an oncogene. Meanwhile, opposite downregulation of STAT4 was reported in HCC and STAT4 may play a suppressive role in liver cancer. The expression and clinical value, as well as its potential mechanism of STAT4 in lung cancer still remain unclarified. Therefore, in the present study, we detected the expression of STAT4 protein and explored its association with relevant clinicopathological features in 365 cases of lung cancer patients.

Materials and methods

Study design

Two pathologists reviewed the collected hematoxylin-eosin stained section of cancer and normal lung tissue for the transfer to the tissue microarray (TMA). Two tissue cores of 0.6 mm in diameter were taken out from each donor block and displayed into a new blank recipient paraffin block (35 mm × 22 mm × 5 mm) with a commercial microarray instrument (Beecher Instruments, USA). Two TMA blocks contained 150 cases (300 tissue cores) and the third one included of 95 cases (190 tissue cores), respectively. Altogether, three blocks were prepared, which consisted of tissues from 365 lung cancer cases and 30 normal lung cases. All lung cancer patients were aged from 19 to 84 years, with an average age of 57.67 years. Tumor cases from the First Affiliated Hospital of Guangxi Medical University, P. R. China between January 2010 and December 2012 were randomly selected, and we also collected normal lung tissues from autopsies without any lung diseases between March 2009 and November 2012 in the same hospital. The age of normal lung cases ranged from 19 to 73 years, with a mean age of 54.03 years. Tumor specimens classified based on histological type consisted of SCLCs (26 cases) and NCSCLs (339 cases), including adenocarcinomas (ADC, 127 cases), squamous cell carcinomas (SCC, 175 cases), adenosquamous carcinomas (28 cases), undifferentiated carcinomas (8 cases), large cell carcinoma (1 case). As for the subtypes of ADC, there were 83 acinar ADC, 19 papillary ADC, 18 lepidic carcinomas and 7 mucinous carcinoma cases (**Table 1**). The study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Guangxi Medical University (NO. 2013-KY-Guoji-016). Informed consent to use the samples for research was written and achieved from the patients and clinical doctors. All the cases were annotated for STAT4 expression by two pathological doctors independently without the knowledge of patient information.

Evaluation of immunostaining

Expression of STAT4 was detected by immunohistochemistry. The labeled streptavidin biotin

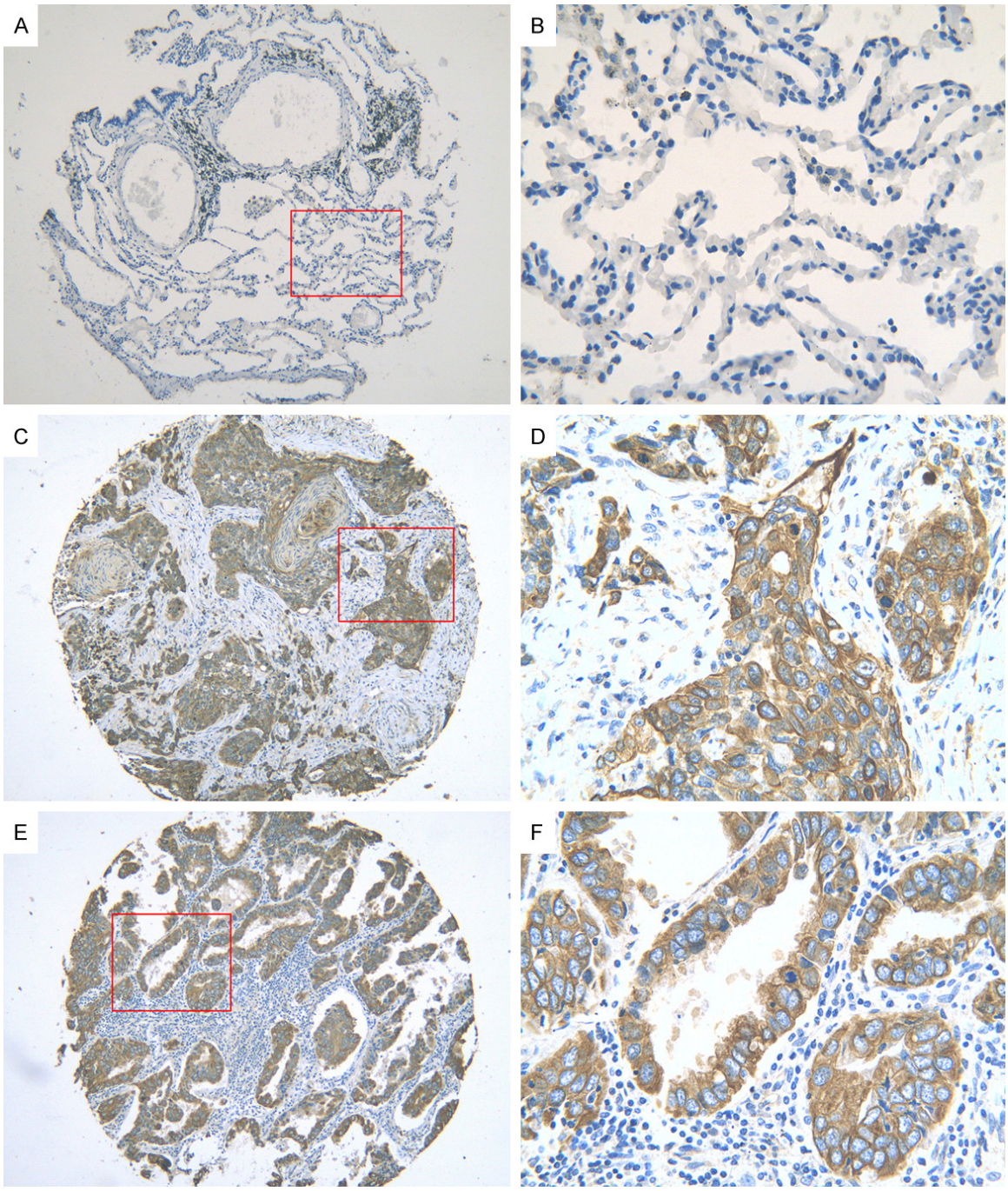

Figure 1. Representative photographs of the immunohistochemical expression of STAT4 in lung tissues. Staining for STAT4 in normal lung tissues (A, × 100; B, × 400), positive staining for STAT4 in lung squamous carcinoma tissues (C, × 100; D, × 400) and lung adenocarcinoma tissues (E, × 100; F, × 400).

method was used on 4-mm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization and antigen retrieval. Mouse monoclonal antihuman STAT4 antibody (PL-68, CA, USA, 1:300 dilution) was purchased from Santa Cruz Biotech Company. Immunohistochemical staining reagents were supplied by Shanghai Changdao Biotech Company. The procedure was conducted following the kit instructions. The average percentage of positive cells was recorded as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). The intensity of

Table 2. Expression of STAT4 protein in lung cancer and normal lung tissues

Tissue				STAT4 negative (n, %)	STAT4 positive (n, %)	n	Z	*P* value
Normal lung tissue				21 (70)	9 (30)	30		
Lung cancer				164 (44.9)	201 (55.1)	365	0.008	0.001
	SCLC			8 (30.8)	18 (69.2)	26	-2.904	0.004
	NSCLC			156 (46)	183 (54)	339	-2.517	0.012
		Adenocarcinoma		59 (46.5)	68 (53.5)	127	-2.313	0.021
			Acinar adenocarcinoma	31 (37.3)	52 (62.7)	83	-3.061	0.002
			Papillary adenocarcinoma	13 (68.4)	6 (31.6)	19	-0.116	0.908
			Lepidic carcinoma	9 (50)	9 (50)	18	-1.371	0.170
			Mucinous carcinoma	6 (85.7)	1 (14.3)	7	-0.832	0.406
		Squamous cell carcinoma		84 (48)	91 (52)	175	-2.222	0.026
		Adenosquamous carcinoma		10 (35.7)	18 (64.3)	28	-2.593	0.010
		Undifferentiated carcinoma		2 (25)	6 (75)	8	-2.283	0.022
		Large cell carcinoma		1 (100)	0	1		

Table 3. The status of STAT4 in SCLC with other clinical pathological parameters in SCLC

SCLC	n	STAT4 negative (n, %)	STAT4 positive (n, %)	Z	*P* value
Gender				-1.627	0.104
Male	21	8 (38.1)	13 (61.9)		
Female	5	0 (0)	5 (100)		
Age (years)				-1.168	0.243
<60	15	6 (40)	9 (60)		
≥60	11	2 (18.2)	9 (81.8)		
Lymph node metastasis				-3.042	0.002
Yes	13	1 (7.7)	12 (92.3)		
No	10	7 (70)	3 (30)		
TNM				-3.004	0.003
I-II	13	8 (61.5)	5 (38.5)		
III-IV	10	0 (0)	10 (100)		
Tumor diameter (cm)				-1.572	0.116
≤7	19	8 (42.1)	11 (57.9)		
>7	4	0 (0)	4 (100)		
Distal Metastasis				0.000	1
Absent	23	8 (34.8)	15 (65.2)		
Present	0	0	0		

SCLC = small cell lung cancer.

staining was scored as 0 (negative), 1 (weak), 2 (moderate), 3 (strong). Final immunohistochemical results for all samples were calculated by multiplying the scores from the percentage and intensity. Scores more than 2 were considered as positive staining.

Statistical analysis

SPSS20.0 was used in the statistical analysis. STAT4 expression difference among tumor histological subtypes, pathological classifications and grading was evaluated by Kruskal-Wallis H. x^2 test was selected to compare STAT4 expression in different groups classified based on the parameters: age, gender, tumor staging (TNM), tumor size, lymph node metastasis and distal metastasis. Spearman's correlation was applied to assess the relationships between STAT4 expression levels and the clinicopathological parameter. Value of STAT4 protein in lung cancer diagnosis was dissected by using Receiver operator characteristic curve (ROC). Results achieved a $P<0.05$ level at two sides was considered statistically significant.

Results

Differential expression of STAT4 between lung cancer and normal lung tissues

STAT4 was weakly expressed in lymphocytes, macrophages in stroma, also some normal bronchial epithelial cells and alveolar epithelial cells. However, much stronger expression was observed in cancer cells (**Figure 1**). Since STAT4 expression was found both in stroma and

Table 4. The status of STAT4 in NSCLC with other clinical pathological parameters in NSCLC

NSCLC	n	STAT4 negative (n, %)	STAT4 positive (n, %)	Z	*P* value
Gender				-0.782	0.434
Male	254	120 (47.2)	134 (52.8)		
Female	85	36 (42.4)	49 (57.6)		
Age (years)				-0.936	0.348
<60	181	79 (43.6)	102 (56.4)		
≥60	158	77 (48.7)	81 (51.3)		
Lymph node metastasis				-6.876	<0.001
Yes	115	23 (20.0)	92 (80.0)		
No	224	133 (59.4)	91 (40.6)		
TNM				-1.914	0.056
I-II	286	138 (48.3)	148 (51.7)		
III-IV	53	18 (34.0)	35 (66)		
Tumor diameter (cm)				-0.728	0.467
≤7	295	138 (46.8)	157 (53.2)		
>7	44	18 (40.9)	26 (59.1)		
Distal metastasis				-2.752	0.006
Absent	323	154 (47.7)	169 (52.3)		
Present	16	2 (12.5)	14 (87.5)		
Pathological grading				-5.642	<0.001
I	39	29 (74.4)	10 (25.6)		
II	92	51 (55.4)	41 (44.6)		
III	130	37 (28.5)	93 (71.5)		

parenchyma, we initially compared the difference of stromal STAT4 expression between distinct groups. No significant variation of stromal STAT4 expression was achieved between noncancerous and cancer tissues, either between ADC and SCC (data not shown). Thus, the STAT4 expression in lung parenchyma and lung cancer cells was focused on in the current study.

Then, STAT4 expression was evaluated separately in SCLC and NSCLC. Statistically significant difference was clarified between normal lung tissues and SCLC (69.2%, 18/26), as well as between normal lung tissues and NSCLC (54%, 183/339). However, no difference was found between the expression of STAT4 protein in SCLC and NSCLC (**Table 2**). Further, the AUC was 0.696 (95% CI 0.555~0.837, P=0.012) for SCLC, and 0.620 (95% CI 0.519~0.721, P=0.029) for NSCLC, respectively.

Since STAT4 showed similar overexpressing pattern in both SCLC and NSCLC groups, we considered these two groups as one integral whole as lung cancer. STAT4 protein expressed positively in 201 of 365 (55.1%) lung cancer cases in the current study, significantly higher than that in the normal lung tissues (30%, 9/30, **Table 2**). Furthermore, the diagnostic value assessment of STAT4 was performed by using ROC curve. The area under curve (AUC) of STAT4 was 0.625 (95% CI 0.525~0.726, P=0.022). The diagnostic sensitivity and specificity of STAT4 were 55.1% and 30.0%, respectively.

Concerning the different histological classifications of NSCLC, STAT4 showed significantly higher expression in ADC (53.5%, 68/127), SCC (52%, 91/175), adenosquamous carcinoma (64.3%, 18/28) and undifferentiated carcinoma (75%, 6/8) (all P<0.05) than that in the normal lung tissues. Nevertheless, no statistically significant difference of STAT4 expression was showed in the above 4 subgroups. When ADC was further divided into different patterns, remarkably higher expression of STAT4 was also found in acinar ADC compared with that in normal lung tissues (P=0.002), however, there was no significant difference of STAT4 expression between acinar ADC, papillary ADC, lepidic carcinomas and mucinous carcinomas (**Table 2**).

Relationship of STAT4 protein and other clinicopathological features in lung cancer

Correlations between STAT4 protein and other clinicopathological parameters in both SCLC and NSCLC tissues were further investigated. All of 10 SCLC patients in advanced stages (III and IV) presented STAT4 positive (100%), remarkably higher than that in the early stages (I and II, 38.5%, **Table 3**). STAT4 was found in 35 out of 53 NSCLC patients in advanced stages (III and IV) expressed positively (66.0%), obviously higher than that in early stages (I-II,

Table 5. The correlation between STAT4 expression and other clinical pathological parameters in lung cancer, SCLC and NSCLC

STAT4 expression		Lung cancer	SCLC	NSCLC
TNM	R value	0.154	0.641	0.104
	P value	0.003	0.001	0.055
Distal Metastasis	R value	0.142	—	0.150
	P value	0.007	—	0.006
Gender	R value	0.057	0.325	0.043
	P value	0.280	0.105	0.435
Age	R value	-0.034	0.234	-0.051
	P value	0.519	0.251	0.350
Tumor size	R value	0.061	0.335	0.04
	P value	0.245	0.118	0.468
Lymph node metastasis	R value	0.395	0.649	0.374
	P value	0.000	0.001	<0.001

148/286, 51.7%, **Table 4**). Tumors with lymph node metastasis (SCLC: 92.3%, 12/13; NSCLC: 80.0%, 92/115) expressed STAT4 protein obviously higher than that with no lymph node metastasis (SCLC: 30%, 3/10; NSCLC: 40.6%, 91/224). Higher STAT4 expression level was also found in distal metastasis tumor group (NSCLC: 87.5%, 14/16) compared with that in the group without distal metastasis (52.3%, 169/323). In addition, statistical significant difference of STAT4 expression level was found between different pathological grades in NSCLC patients, the rate of STAT4 expressed positively in grade I (25.6%, 10/39) and grade II (44.6%, 41/92) tumor tissues was significantly lower than that in grade III (71.5%, 93/130, P<0.001, **Table 4**). Spearman test showed there were consistent correlations between STAT4 expression and TNM (r=0.641, P=0.001) and lymph node metastasis (r=0.649, P=0.001) in SCLC group (**Table 5**). As to 339 NSCLC patients, there were also relationships between STAT4 protein expression and lymph node metastasis (r=0.374, P<0.001), distal metastasis (r=0.150, P=0.006, **Table 5**). We also analyzed the expression level divergence of STAT4 in the corresponding groups of gender, age and tumor diameter, no obvious difference was observed in any of the 3 groups (**Tables 3**-**5**).

Similar to the individual groups of SCLC and NSCLC, the trend of STAT4 expression was also observed in the total lung cancer population as one ensemble. In advanced tumor stages III and IV, the rate of STAT4 protein expression (71.4%, 45/63) was remarkably higher than that in early stages (I and II, 51.2%, 153/299). Higher level of STAT4 was found in the group with metastatic lymph node (104/128, 81.2%) than that without (94/234, 40.2%). The positive ratio of STAT4 expression was 87.5% (14/16) in the patients with tumor distal metastasis, significantly higher than those without (53.2%, 184/346, **Table 6**). STAT4 protein expression was positively associated with TNM stages (r=0.154, P=0.003), lymph node metastasis (r=0.395, P< 0.001) and distal metastasis (r=0.142, P=0.007) by using spearman correlation. However, there was no significant correlation between STAT4 protein expression and other clinicopathological parameters, for instance, gender, age and so on (**Table 5**).

Discussion

In the present study, we detected higher expression of STAT4 in 365 lung cancer than that in 30 normal lung tissues. Then we continued to observe the correlation of STAT4 expression with some related clinical pathological parameters in SCLC, NSCLC separately, as well as in lung cancer population as a whole.

STAT4 is a member of the STAT family that regulates various genes transcription including IFN-c by the JAK/STAT pathway [11]. Currently, STAT genes have been clarified to play important roles in the progression of various tumor tissues, including gastrointestinal cancer, breast cancer, lymphoma, Kaposi sarcoma [12-15]. Meanwhile, STAT protein, as a transcription factor, regulates cell proliferation, differentiation and apoptosis from molecular level in lung inflammation, it plays an essential role in disease, tumor, and immune diseases [16]. STAT4 has been reported to be prognostic indicator in patients with hepatocellular carcinoma (HCC) [10]. However, there has been no study to explore the significance of STAT4 expression levels in lung cancer patients.

As for the STAT4 expression distinction between cancer and non-cancerous tissues, Wubetu et al performed a study with HCC tissues [10]. Wubetu et al used quantitative real-time poly-

Table 6. Differential expression of STAT4 protein of other clinicopathological parameters in lung cancer

Lung cancer	n	STAT4 negative (n, %)	STAT4 positive (n, %)	Z	*P* value
Gender				-1.082	0.279
Male	275	128 (46.5%)	147 (53.5%)		
Female	90	36 (40%	54 (60%)		
Age (years)				-0.646	0.518
<60	196	85 (43.4%)	111 (56.6%)		
≥60	169	79 (46.7%)	90 (53.3%)		
TNM				-2.932	0.003
I-II	299	146 (48.8)	153 (51.2)		
III-IV	63	18 (28.6)	45 (71.4)		
Lymph node metastasis				-7.496	<0.001
Yes	128	24 (18.8)	104 (81.2)		
No	234	140 (59.8)	94 (40.2)		
Tumor diameter (cm)				-1.165	0.244
≤7	48	18 (46.5)	30 (53.5)		
>7	314	146 (37.5)	168 (62.5)		
Distal metastasis				-2.692	0.007
Absent	346	162 (46.8)	184 (53.2)		
Present	16	2 (12.5)	14 (87.5)		

merase chain reaction to clarify that STAT4 expression level was significantly lower in HCC tissue than corresponding non-tumor tissue. Surprisingly, remarkable contrast of STAT4 expression level was observed between lung cancer and HCC. The present study showed that higher level of STAT4 protein was expressed in total lung cancer population compared with the normal lung tissues. Furthermore, ROC curve suggested that STAT4 expression had moderate correlations with lung cancer clinical parameters.

Identical to the total lung cancer group, SCLC and NSCLC tissues also expressed STAT4 significantly higher than normal lung tissues and the moderate diagnostic values of STAT4 high expression for SCLC and NSCLC were verified by ROC curves. Obvious differences between SCLC tissues and NSCLC tissues have not been found in our study. The datasets of lung cancer cell lines from Gemma et al and Miyanaga et al [17, 18] provided the concordant results. In their reports, the authors compared the STAT4 mRNA level with microarray assay and found no significant difference between SCLC (n=10) and NSCLC (n=19) cell lines [17, 18]. Furthermore, no difference of STAT4 mRNA was noted between SCC (n=9) and ADC (n=10) cell lines, which supported the current finding based on STAT4 protein expression between 175 cases of lung SCC and 127 cases of ADC. It has been proved that STAT4 impairment resulted in decreased antitumor activity [19], however, the molecular mechanism of high STAT4 expression in lung cancer remains unknown. The current result indicated that high expression of STAT4 in lung tissues promotes tumor incidence and STAT4 may be a promising biomarker in the diagnosis for lung cancer.

In the current study, statistical analysis suggested STAT4 could influence tumor progression in a variety of means in lung cancer. The positive rate of STAT4 expression was significantly higher in advanced tumors as compared with that in early stage tumors. The cases with lymph node metastasis expressed higher STAT4 than those with no metastasis. Above clinical features are crucial factors for the outcome of tumor patients. It has been shown that low expression of STAT4 was associated with large tumor size, advanced TNM stage, presence of vascular invasion, as well as moderate or poorly differentiated tumors in the HCC [20]. All studies on relationship of STAT4 with HCC clinical parameters supported that STAT4 might be a repressor of HCC metastasis and invasion

[10, 21, 22]. Their study suggested that STAT4 influenced tumor progression via regulating IFN-c production positively, which was negatively correlated with HCC development including metastasis, tumor stages [23, 24]. Interestingly, our finding showed that high expression of STAT4 has more clear significance in lung tumor progression. The result implied that STAT4 might be a promoter of lung cancer progression opposite to that of HCC.

STAT4 had been reported to express in many activated immune cells [25]. In addition, several types of autoimmune diseases or cancers are led by STAT4 expression via the inflammatory response, regulation of T cell differentiation and production of IFN-y, a crucial cytokine for hepatocyte apoptosis, liver regeneration, viral limitation and tumor suppression [19]. Activated STAT4 by IL-12 and IL-23 forms dimers and relocates to the nucleus to regulate its target genes, especially IFN-γ [26, 27]. Michael J et al had proposed that STAT4 deficiency results in the production of interferon-γ which is critical in anti-tumor activities. In addition, STAT4 was showed to be targeted protein for miR-141 in inhibiting gastric malignancy invasion and metastasis [4]. Their study showed miR-141 expression was down-regulated, while STAT4 expression was up-regulated in gastric cancer, miR-141 inhibited gastric tumor progression by repressing STAT4 expression, which indicated that STAT4 high expression play important role in gastric tumor invasion and metastasis. However, our group reported that miR-141 was also up-regulated in lung cancer [28], inconsistent with that in gastric cancer. Litvinov et al also reported that up regulation of STAT4 in cutaneous T-cell lymphoma (CTCL) patients was resulted from miR-155 inhibition [6]. So that STAT4 may play functional role in lung cancer tumor progression different from gastric malignancy and lymphoma.

In conclusion, expression level of STAT4 was positively correlated with histology grade, lymph node metastasis, TNM stage, distal metastasis of lung cancer, which suggested STAT4 might play a crucial role in lung cancer tumorigenesis and progression. We firmly believe that thorough study about STAT4 expression in lung cancer can reveal a novel strategy for the diagnosis and prognosis prediction of lung cancer. Apart from this, it may provide another route of molecular targeted therapy for NSCLC patients.

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Disclosure of conflict of interest

None.

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