SPANDIDOS PUBLICATIONS EXPERIMENTAL AND THERAPEUTIC MEDICINE 15: 2837-2843, 2018

Girdin protein: A potential metastasis predictor associated with prognosis in lung cancer

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Received July 23, 2016; Accepted May 5, 2017

DOI: 10.3892/etm.2018.5773

Abstract. The present study explored the relationship between Girdin protein expression and the survival rate of patients with lung carcinoma. A total of 334 lung cancer specimens, 20 benign lung disease tissue sections and 24 fresh tissues from patients with lung carcinoma were analyzed by immunohistochemistry and western blotting. Girdin protein was expressed in 130/334 (38.93%) of the cases examined. Girdin protein expression was correlated with tumor/node/metastasis stage (P<0.001), lymph node metastasis (P=0.001), distant metastasis (P<0.001) and specimen sites (P=0.034). Girdin expression was also correlated with signal transducer and activator of transcription 3 (STAT3) expression (P<0.001). Patients with high Girdin and STAT3 expression had a significantly poorer prognosis compared with those with low/high, high/low or low/low expression (P<0.001). In summary, Girdin may be a prognostic marker of lung cancer and serve as a biomarker for metastasis.

Introduction

According to the National Cancer Institute, although lung cancer mortality rates have declined due to reduced tobacco use, lung cancer remains the leading cause of cancer-related mortality worldwide (1). A high rate of metastasis at diagnosis is the principal reason for poor prognosis (2). Tumor invasion and metastasis are complex and dynamic processes controlled by multiple factors, including tumor cells and the tumor microenvironment (3). Lung cancer patients are prone to distant metastases; thus, the survival period is shortened and life quality is affected (4). Nevertheless, the mechanism of metastasis in lung cancer remains unclear.

The phosphoinositide 3-kinase-protein kinase B (Akt) signaling pathway is believed to be closely associated with metastasis (5). Girdin, an Akt substrate and newly discovered nuclear actin-binding protein, has a key role in promoting cell migration and angiogenesis during embryonic development, inflammation and tumor angiogenesis, and it is highly expressed in several human malignant carcinomas, such as colon, breast, glioblastoma and esophageal carcinomas (6-12). A study by Song *et al* (13) assessed Girdin expression and the correlation between its expression and clinical-pathological parameters and survival in a cohort of 36 consecutive patients with non-small cell lung cancer (NSCLC), and observed a significant correlation between elevated Girdin expression and blood vessel infiltration of the tumor.

The Janus kinase-signal transducer and activator of transcription (STAT) signaling pathway is also closely associated with many biological processes, particularly metastasis (14). The STAT family consists of six members (STAT1-STAT6), of which STAT3 is one of the most common sustained activated signaling proteins (15). A study by Dunkel *et al* (16) demonstrated that Girdin is capable of forming a positive feedback loop to increase the activity of STAT3, thereby promoting tumor invasion and migration. In a previous study, to explore whether Girdin is mediated by STAT3 in lung cancer, the authors of the present study depleted endogenous STAT3 and observed that Girdin expression decreased (17). It was also found that interleukin (IL)-17 promotes tumor angiogenesis in NSCLC by activating STAT3/Girdin signaling in NSCLC cell lines, which subsequently upregulates vascular endothelial growth factor (17). Nevertheless, few studies have explored the expression of Girdin protein and STAT3, as well as their relationship with lung cancer.

In the present study, the correlation between Girdin protein and STAT3 protein in lung cancer was evaluated using immunohistochemistry (IHC). A prognostic model based on clinical parameters was also generated to determine whether Girdin could act as a prognostic biomarker for lung cancer.

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Key words: lung cancer, immunohistochemistry, western blotting, Girdin, signal transducer and activator of transcription 3

Patients and methods

Patient tissue samples. A total of 334 NSCLC tissue sections, 20 benign lung disease tissue sections, 20 adjacent normal lung tissues sections, 24 fresh NSCLC tissues and 5 fresh normal lung tissue sections were obtained with informed consent at the Harbin Medical University Cancer Hospital (Harbin, China) between January 2005 and December 2006. All patients included in the present study had been surgically resected and diagnosed with stage I-IIIA NSCLC. Patients with any other types of cancer, or who missed follow-up appointments were excluded from the study. This retrospective analysis was approved by the Ethics Committee of Harbin Medical University Cancer Hospital. The clinical parameters extracted from medical records included: Age; sex; smoking history; Eastern Cooperative Oncology Group (ECOG) performance status (18); histological type and grade; stage (IASLC 7th TNM Staging system) (19); metastasis sites; diameter of the carcinoma; and specimen sites.

IHC. For IHC, 4-μm-thick formaldehyde-fixed (fixed with 4% formaldehyde at room temperature for 24 h), paraffin-embedded sections of 334 NSCLC, 20 benign lung disease and 20 adjacent normal lung tissues were deparaffinized in xylene and then rehydrated in serially graded alcohols. Antigens were retrieved by boiling the samples in 10 mM sodium citrate buffer at pH 6.0 for 30 min. Subsequently, the sections were washed with phosphate-buffered saline (pH 7.4), blocked with 3% hydrogen peroxide at room temperature for 20 min and incubated overnight at 4˚C with anti-Girdin (ab111035; 1:100; Abcam, Cambridge, UK) and anti-STAT3 (ab119352; 1:500; Abcam) antibodies. The slides were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibodies (SC2040, 1:400, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 30 min at room temperature, followed by signal detection with diaminobenzidine. The slides were counterstained with hematoxylin at room temperature for 5 min. The mean percentage of positive tumor cells was determined in at least five fields at magnification, x200 using a light microscope.

The slides were evaluated independently by two experienced pathologists who reached a consensus. The percentages of positive cells were categorized as follows: 0, 0%; 1, 0-10%; 2, 10-50%; and 3, >50%. The staining intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The scores for the percentage of positive cells and staining intensity were multiplied to achieve a weighted score for each case. Cases with scores ≤4 were defined as low expression and cases with scores >4 were defined as high expression.

Western blot analysis. A total of 24 fresh NSCLC and 5 normal tissues were washed three times with PBS solution and treated by ultrasonic lysis with a radioimmunoprecipitation lysis buffer (P0013C; Beyotime Institute of Biotechnology, Haimen, China) for protein extraction. Protein were quantified by BCA. A total of 30 μg of protein were loaded per lane and separated by 10% SDS-PAGE, after which the proteins were transferred to a polyvinylidene difluoride membrane. Subsequently, the membrane was blocked with 5% skim milk for 1 h at room temperature and incubated with primary antibodies directed against Girdin, (ab113890; 1:500; Abcam) and β-actin (4970P; 1:1,000; CST Biological Reagents Co., Ltd., Shanghai, China) overnight at 4˚C. Appropriately diluted specific secondary antibodies (anti-rabbit IgG; ZB2301; 1:1,000; OriGene Technologies, Inc., Beijing, China) were added and incubated for 1 h at room temperature. An enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to detect and analyze immunostained protein bands using a charge-coupled camera (LAS4000; Fujifilm, Tokyo, Japan) and Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Table I. Correlation between Girdin and STAT3 expression in non-small cell lung cancer tissues.

	Girdin			
Transcription factor	High	Low	*r*	P-value
STAT3				
High	110	29	0.696	<0.001
Low	20	175		

STAT3, signal transducer and activator of transcription 3.

Statistical analysis. Data were presented as the mean ± standard deviation. Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference. ANOVA and Dunnett's post hoc test was performed for the comparison of Girdin expression between fresh tumor and normal tissues. A multivariate Cox regression model was used to analyze prognostic variables for survival measures. Chi-squared tests were used to examine the statistical association between clinical-pathological and IHC data. Survival curves were plotted using the Kaplan-Meier method and differences were assessed using the log-rank test. The correlation between Girdin and STAT3 was calculated using Spearman's rank correlation coefficient.

Results

Patient characteristics. To evaluate the clinical significance of Girdin expression in NSCLC, an IHC analysis of 334 NSCLC tissues samples, 20 benign lung disease tissues (pulmonary hamartoma, pulmonary fibroma, pulmonary hemangioma and pneumonia) and 20 adjacent normal lung tissues was performed. The mean age of the 334 NSCLC patients enrolled in the present study was 50.87 years (range, 29-80 years). Of these, 178/334 (53.23%) patients had lymph node metastasis and 82/334 (24.53%) exhibited distant metastasis (Table I).

Expression and localization of Girdin and STAT3 in NSCLC, benign lung disease and normal lung tissues. As demonstrated in Fig. 1, cytoplasmic and membrane Girdin immunoreactivity was detected in 130/334 lung cancer samples (38.93%) and 10% (2/20, one pulmonary hamartoma and one pulmonary hemangioma) of benign cases, whereas it was not present in the adjacent normal tissues (0/20). STAT3

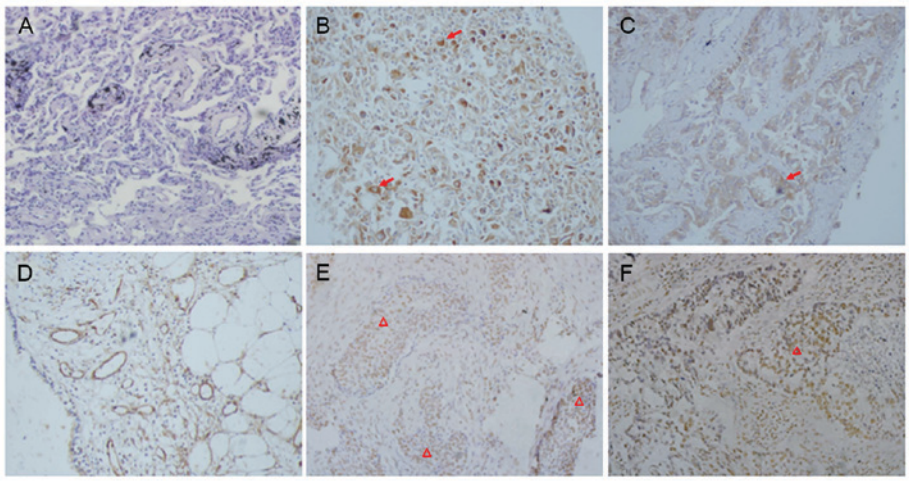

Figure 1. Immunohistochemical staining of Girdin and STAT3 in non-small cell lung cancer, benign lung disease and normal lung tissues (magnification, x200;). (A) Negative staining of Girdin in normal lung tissues. (B) Positive staining of Girdin in lung squamous cell carcinoma. (C) Positive staining of Girdin in lung adenocarcinoma. (D) Positive staining of Girdin in pulmonary hamartoma. (E) Positive staining of STAT3 in lung squamous cell carcinoma. (F) Positive staining of STAT3 in lung adenocarcinoma. Red arrows indicate examples of cytoplasmic and membrane staining; red triangles indicate examples of nucleic staining. STAT3, signal transducer and activator of transcription 3.

was predominately localized in the nuclei of tumor cells. Western blotting was used to investigate Girdin expression in fresh tumor tissues and normal tissues (Fig. 2). Girdin expression was significantly higher in several NSCLC tissue samples compared with normal tissues as determined by one-way ANOVA ($P<0.05$; Fig. 2).

The potential correlation between the expression of Girdin and STAT3 in NSCLC was assessed. Spearman's rank correlation analysis revealed that Girdin expression was closely correlated with STAT3 expression in the NSCLC patient cohort ($r=0.696$; $P<0.001$) (Table I).

Relationship between Girdin and STAT3 overexpression and clinical-pathological parameters. The correlations between Girdin and STAT3 expression and the clinicopathological characteristics of NSCLC are demonstrated in Table II. Lung tumor expression of Girdin was not dependent on age, sex, smoking history, family history, histology, ECOG performance status, histopathological subtype, degree of differentiation, tumor size, metastatic site or T stage. Lung tumor expression of STAT3 in the lung cancer cases was not dependent on age, sex, smoking history, family history, histology, ECOG performance status, histopathological subtype, degree of differentiation or specimen sites. Elevated expression of Girdin was associated with positive lymph node metastasis status ($P=0.001$), positive distant metastasis status ($P<0.001$), later TNM stage ($P<0.001$) and more tumor sites ($P=0.034$). Elevated expression of STAT3 was correlated with later TNM stage ($P=0.007$), positive lymph node metastasis status ($P<0.001$), positive distant metastasis ($P=0.011$), later T stage ($P=0.004$) and larger tumor diameter ($P=0.002$).

Elevated Girdin and STAT3 expression is associated with poor prognosis in NSCLC. The Kaplan-Meier survival curves of Girdin and STAT3 for overall survival (OS) and progression-free survival (PFS) are demonstrated in Fig. 3. Patients with elevated Girdin expression were observed to have significantly shorter OS ($P<0.001$) and PFS ($P<0.001$) compared with those with lower expression. Patients with increased STAT3 expression were observed to have significant shorter OS ($P<0.001$) and PFS ($P<0.001$) rates compared with patients with low-level expression. Furthermore, patients with low expression of both Girdin and STAT3 were observed to have significantly longer OS ($P<0.001$) and PFS ($P<0.001$) compared with individuals with high/high expression and others (low/high, high/low) expression (Fig. 4).

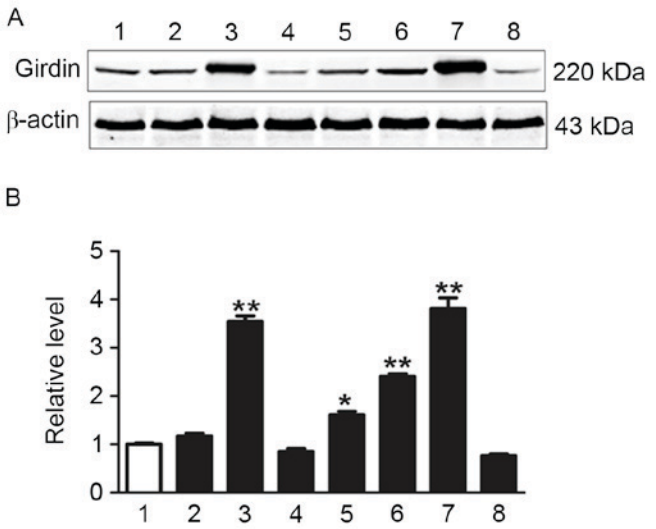

Figure 2. Western blot analysis of Girdin in non-small cell lung cancer and normal lung tissues. (A) Western blot analysis in representative samples of NSCLC and normal tissues and (B) densitometry analysis of protein expression relative to normal tissue. 1, normal tissue; 2-8, NSCLC tissues. Girdin expression was significantly higher in several NSCLC tissue samples (3,5,6 and 7) compared with normal tissues. $^{*}P<0.01$ and $^{**}P<0.001$ vs. normal tissue.

Elevated Girdin expression is independently associated with OS and PFS in NSCLC. To identify prognostic variables of NSCLC, a multivariate analysis was performed. It was

Table II. Association between Girdin, STAT3 and clinicopathological factors in non-small cell lung cancer (n=334).

	Girdin expression			STAT3 expression		
Parameter	Low	High	P-value	Low	High	P-value
Age, years			0.625			0.822
<55	68	40		64	44	
≥55	136	90		131	95	
Gender			0.508			0.223
Male	142	86		128	100	
Female	62	44		67	39	
Family history			0.448			0.094
Yes	40	30		47	23	
No	164	100		148	116	
Smoking status			0.473			0.616
Non-smoker	118	70		112	76	
Smoker	86	60		83	63	
ECOG status			0.799			0.158
0-1	190	122		179	133	
≥2	14	8		16	6	
Histology grade			0.104			0.633
Well-differentiated	2	2		2	2	
Moderately differentiated	62	26		55	33	
Poorly differentiated	140	102		138	104	
Histological type			0.496			0.764
Adenocarcinoma	106	64		96	74	
Squamous cell carcinoma	80	58		83	55	
Other	18	8		16	10	
TNM stage			<0.001			0.007
I-IIIA	150	56		132	74	
IIIB-IV	54	74		63	65	
Tumor stage			0.693			0.004
T1	44	26		48	22	
T2	114	72		108	78	
T3	26	22		18	30	
T4	20	10		21	9	
Lymph node metastasis			0.001			<0.001
Yes	94	84		88	90	
No	110	46		107	49	
Distant metastasis			<0.001			0.011
Yes	26	56		38	44	
No	178	74		157	95	
Diameter of tumor, cm			0.434			0.002
≤3	58	32		64	26	
3-7	134	86		123	97	
>7	12	12		8	16	
Sites of specimen			0.034			0.797
Primary tumor site	190	112		177	125	
Metastasis tumor site	14	18		18	14	

ECOG, Eastern Cooperative Oncology Group; TNM, tumor/node/metastasis; STAT3, signal transducer and activator of transcription 3.

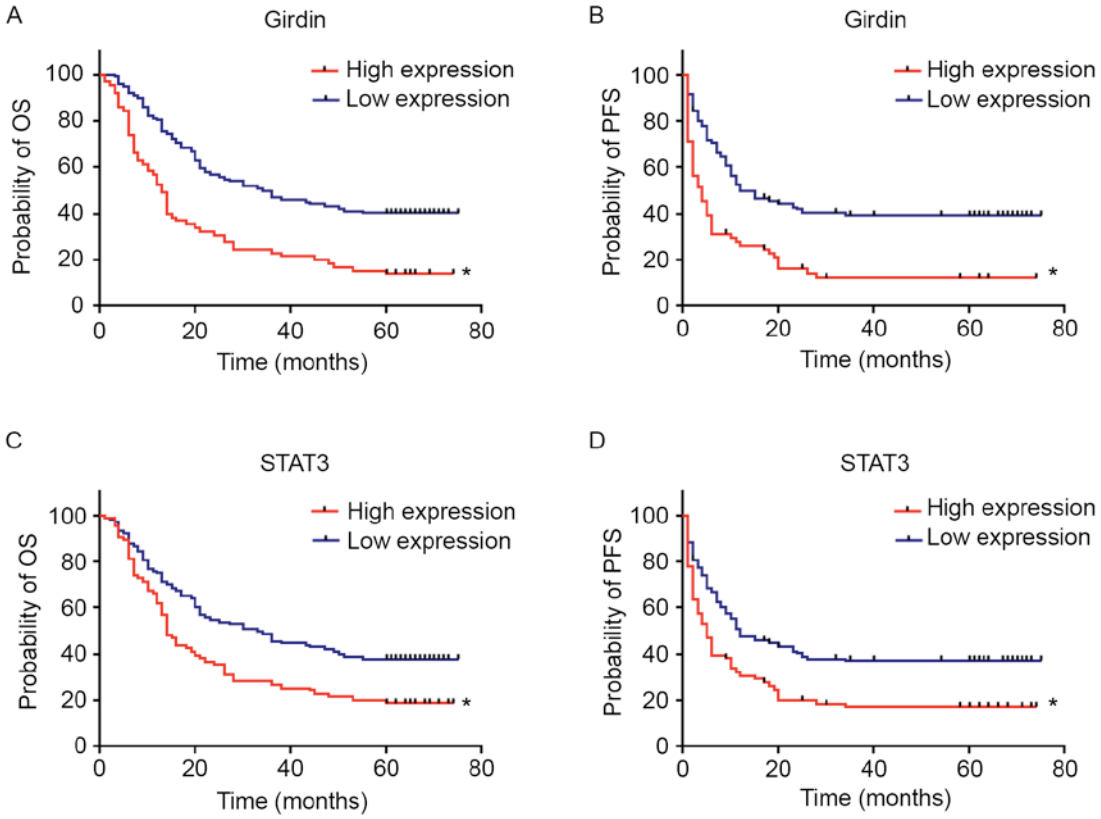

Figure 3. Kaplan-Meier survival curves of Girdin and STAT3 expression for OS and PFS in patients with non-small cell lung cancer. Patients with high Girdin expression were observed to have poorer (A) median OS (13 vs. 35 months) and (B) median PFS (4 vs. 12 months) compared with those with lower expression. Patients with high STAT3 expression were observed to have poorer (C) median OS (14 vs. 33 months) and (D) median PFS (5 vs. 12 months) compared with those with lower expression. *P<0.001 vs. low expression. OS, overall survival; PFS, progression-free survival; STAT3, signal transducer and activator of transcription 3.

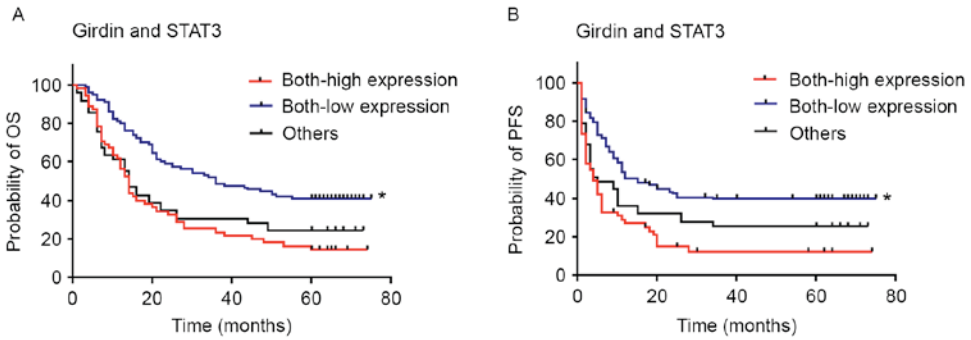

Figure 4. Kaplan-Meier survival curves of combined Girdin and STAT3 expression for OS and PFS in patients with non-small cell lung cancer. (A) Patients with low expression of both Girdin and STAT3 were observed to have significantly longer median OS compared with others and high/high expression (35 vs. 14 vs. 14 months). (B) Patients with low expression of both Girdin and STAT3 were observed to have significantly longer median PFS compared with others and high/high expression (12 vs. 5 vs. 4 months). *P<0.001 vs. both high expression and others. OS, overall survival; PFS, progression-free survival; STAT3, signal transducer and activator of transcription 3; others, low/high or high/low expression of Girdin and STAT3, respectively.

identified that TNM stage (P=0.002 and P=0.001), lymph node metastasis (P=0.009 and P=0.004), distant metastasis (P=0.048 and P=0.001) and Girdin expression (P=0.004 and P=0.001) were prognostic factors in NSCLC for OS and PFS, respectively (Table III).

Discussion

Currently, the expression status of Girdin protein and its prognostic value for lung cancer are unclear. In the present study, it was identified that Girdin expression is significantly associated with TNM stage and tumor metastasis in human lung cancer.

Girdin, which is phosphorylated following epidermal growth factor stimulation and is a novel Akt substrate, is essential for cell metastasis (20). It is an important factor for the leading edge of cell pseudopods involved in cell movement (21). A study by Garcia-Marcos *et al* (22) reported that the survival rate of patients with colon cancer and Girdin-positive expression was reduced compared with Girdin-negative expression. Girdin expression also predicted mortality risk,

Table III. Multivariate survival analysis of OS and PFS in patients with non-small cell lung cancer (n=334).

	OS			PFS		
Parameter	HR	95% CI	P-value	HR	95% CI	P-value
TNM stage						
I-IIIA	1.000			1.000		
IIIB-IV	0.490	0.316-0.757	0.002	0.480	0.311-0.739	0.001
Tumor stage						
T1	1.000			1.000		
T2	0.454	0.251-0.821	0.009	0.449	0.249-0.810	0.008
T3	0.901	0.544-1.491	0.684	0.912	0.551-1.511	0.722
T4	1.303	0.736-2.307	0.363	1.780	1.009-3.141	0.047
Lymph node metastasis						
Yes	1.000			1.000		
No	1.462	1.101-1.942	0.009	1.505	1.139-1.989	0.004
Distant metastasis						
Yes	1.000			1.000		
No	1.561	1.005-2.427	0.048	2.116	1.355-3.311	0.001
Girdin expression						
Low	1.000			1.000		
High	1.894	1.228-2.921	0.004	2.127	1.366-3.311	0.001
STAT3 expression						
Low	1.000			1.000		
High	0.796	0.527-1.202	0.277	0.686	0.445-1.058	0.088

HR, hazard ratio; CI, confidence interval; OS, overall survival; PFS, progression-free survival; TNM, tumor/node/metastasis; STAT3, signal transducer and activator of transcription 3.

independent of microsatellite stability status. The authors concluded that Girdin may serve as a convenient metastasis biomarker for colon cancer (22). In the present study, it was demonstrated that patients with elevated Girdin expression had poorer OS and PFS compared with those with lower expression levels. These results are consistent with previous investigations of Girdin in other cancer types. In breast cancer tissues and cell lines, Girdin was highly expressed, and the co-expression of Girdin and tumor necrosis factor receptor 4 led to an increased rate of lymph node metastasis (23). A study by Nishimae *et al* (24) reported that the expression of Girdin in invasive breast cancer was strongly associated with lymph node metastasis. In esophageal squamous cell carcinoma (ESCC), Girdin was demonstrated to be involved in the motility of ESCC cells, and the expression of Girdin protein was inversely correlated with ESCC patient survival (12). In the present study, it was identified that the expression rate of Girdin in NSCLC was 38.93%, which differed from 72.2% (26/36) in a study by Song *et al* (13) of 36 NSCLC patients undergoing surgery. This difference may be because 334 patients with different stages were recruited to the present study, whereas only patients with early-stage disease were enrolled in the study by Song *et al* (13). The present study also demonstrated that tissues with stronger expression of Girdin were obtained from metastasis sites, which may be because Girdin facilitates cell invasion and metastasis.

STAT3 is a STAT family member activated by tyrosine phosphorylation in response to various factors, such as epidermal growth factor and IL-6 (25,26). It was reported by Dunkel *et al* (16) that STAT3 protein upregulates Girdin expression, and that Girdin enhances STAT3 activation in a positive feedback loop during wound healing and tumor metastasis. STAT3 was also demonstrated to be essential for Girdin expression under stimulated tension force under physiological conditions, as well as for osteoblast proliferation and migration during quiescence (27). These findings suggest that STAT3/Girdin pathway activation has a critical role in proliferation and migration. In the present study, it was revealed that Girdin overexpression was correlated with STAT3 in patient tissues. The results indicated that patients with high-level expression of both Girdin and STAT3 had lower OS and PFS rates compared with low/high, high/low and low/low expression, which indicates that STAT3/Girdin may serve an essential role in malignant behavior in NSCLC.

In conclusion, the present data indicated that Girdin may be a biomarker for metastasis in patients with NSCLC. Combined Girdin and STAT3 expression could predict poor prognosis in patients with NSCLC.

Acknowledgements

The present study was supported by the Education Department Foundation of Heilongjiang Province (grant no. 12521313).

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