RESEARCH COMMUNICATION

Clinical Significance of SH2B1 Adaptor Protein Expression in Non-small Cell Lung Cancer

Hang Zhang[1], Chao-Jun Duan[2], Wei Chen[1], Shao-Qiang Wang[1], Sheng-Kang Zhang[1], Shuo Dong[1], Yuan-Da Cheng[1], Chun-Fang Zhang[1]*

Abstract

The SH2B1 adaptor protein is recruited to multiple ligand-activated receptor tyrosine kinases that play important role in the physiologic and pathologic features of many cancers. The purpose of this study was to assess SH2B1 expression and to explore its contribution to the non-small cell lung cancer (NSCLC). Methods: SH2B1 expression in 114 primary NSCLC tissue specimens was analyzed by immunohistochemistry and correlated with clinicopathological parameters and patients' outcome. Additionally, 15 paired NSCLC background tissues, 5 NSCLC cell lines and a normal HBE cell line were evaluated for SH2B1 expression by RT-PCR and immunoblotting, immunofluorescence being applied for the cell lines. Results: SH2B1 was found to be overexpressed in NSCLC tissues and NSCLC cell lines. More importantly, high SH2B1 expression was significantly associated with tumor grade, tumor size, clinical stage, lymph node metastasis, and recurrence respectively. Survival analysis demonstrated that patients with high SH2B1 expression had both poorer disease-free survival and overall survival than other patients. Multivariate Cox regression analysis revealed that SH2B1 overexpression was an independent prognostic factor for patients with NSCLC. Conclusions: Our findings suggest that the SH2B1 protein may contribute to the malignant progression of NSCLC and could offer a novel prognostic indicator for patients with NSCLC.

Keywords: SH2B1 - non-small cell lung cancer - metastasis - prognosis

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Introduction

Lung cancer is one of the most common malignant tumors, and a leading cause of cancer death worldwide (Reddy et al., 2011). The occurrence and development of lung cancer is a complex pathological process, including the activation of proto-oncogene, the inactivation of anti-oncogene, DNA repairing genes' mutation and polymorphism, the abnormity of signal transduction. Specially tyrosine kinase receptor signaling pathway is one of most important cell signal transductions in these molecular mechanisms, modulates a series of the cellular physiological and pathological processes.

SH2B1 (once called SH2-B or PSM) belongs to a family of adapter proteins that also in-clude SH2B2 (APS) and SH2B3 (Lnk) (Iseki et al, 2000; Yousaf et al., 2001), which is first identified as an FcεR1 immunoreceptor tyrosine-based activation motif-interacting protein (Osborne et al., 1995). Now that four splice variants of SH2B1 have been identified, α, β, γ and δ, which have identical N-terminal and Src homology 2 (SH2) domains but differ only in their C-terminal domains (Osborne et al., 1995; Rui et al., 1997; Yousaf et al., 2001). Major concentrated research hotspot on SH2B1 β was originally identified through its association with JAK2, a cytoplasmic tyrosine kinase that mediates cytokine actions (Rui et al., 1997). The adapter protein SH2B1 β has been shown to be recruited to multiple ligand-activated receptor tyrosine kinases, including the receptors for nerve growth factor receptor (NGF) (Qian et al., 1998; Rui et al., 1999), insulin-like growth factor-I(IGF-I) (Wang et al., 1998), platelet-derived growth factor (PDGF) receptor (Yousaf et al., 2001), brain-derived neurotrophic factor receptor (Suzuki et al., 2002), glial cell–derived neurotrophic factor (GDNF) (Zhang et al., 2006), as well as fibroblast growth factor receptor(FGFR) (Kong et al., 2002), multiple hormones including growth hormone, leptin and insulin (Rui et al., 1997; Qian et al., 1998; Herrington et al., 2000; Duan et al., 2004; Ren et al., 2005) and the cytokine receptor-associated JAK family kinases (Osborne et al., 1995; Ren et al., 1997; Suzuki et al., 2002).

All the four isoforms of SH2B1 have been shown to be able to increase DNA synthesis and cellular proliferation stimulated by different levels of PDGF or IGF-I induced mitogenesis (Riedel et al., 2000; Yousaf et al., 2001), PDGF and IGF-I receptor are overexpression in the NSCLC tissues and cell lines (Soderdahl et al., 1988; Cosaceanu et al., 2005), they can promote the proliferation

*[1]Department of Cardiothoracic Surgery Xiangya Hospital, [2]Medical Science Institute, Xiangya Hospital, Central South University, Changsha, China *For correspondence: chunfangzhangcn@163.com*

of tumor cells and the formation of new vessels, induce the tumor growth, local infiltration and transfer, also induce vasa lymphatica growth and promote lymph node metastases (Favoni et al., 1994; Koukourakis et al., 1997). SH2B1 β as a novel FGFR3 binding partner that mediates downstream biological effects of FGFR3 activation, increases the phosphorylation and nuclear translocation of STAT5B (Kong et al., 2002). More importantly, activating mutations in FGFR3 have been linked to cancers such as multiple myeloma, cervical carcinoma and bladder cancer (Chesi et al., 1997; Cappellen et al., 1999; Oneyama et al., 2011).

Further Ahmad et al. (2011) indicate that activation of FGFR3 can cooperate with K-Ras and β-catenin mutations in mice to promote lung tumorigenesis so as to contribute to lung cancer. SH2B1 enhances leptin signaling by both JAK2 Tyr813 phosphorylation dependent and independent mechanisms. A great deal of research shows leptin may promote a variety of tumor cell lines' growth, differentiation, proliferation, increase the tumor invasion force and promote the formation of new vessels (Tsuchiya et al., 1999; Somasundar et al., 2004). By acting through the continuing activation of JAK/STAT signaling pathway induces the cellular abnormal proliferation and the malignant transformation (Bahrenberg et al., 2002), leptin induces lung cancer cells' STAT3 phosphoric acid and stimulates lung cancer cells' proliferation that promote the development of the lung cancer (Zhang et al., 2007). The continuing activation of STAT3 also induces the cellular abnormal proliferation and the malignant transformation, so that participate in the occurrence, development and evolution of human malignant neoplasms (Garcia et al., 1998), currently it is considered a cancer gene. Seki Y found STAT3 expressed clearly higher level in lung cancer tissues that was achieved by JAK/ STAT signaling pathways (Seki et al., 2004). SH2B1 β would be predicted to act in a positive feedback manner to increase signals by cytokines that activate JAK2. Though activation of JAKs by cytokine receptor ligands is generally rapid and transient, constitutive activation of JAKs has been observed in a variety of cancers. SH2B1 β through the SH2 domain binds to RET isoforms and its oncogenic derivatives to protect RET from dephosphorylation by protein tyrosine phosphatases, and perhaps represent a likely mechanism contributing to its upregulation that is a crucial initiating event in thyroid cancers (Donatello et al., 2007). Excessive activation of RET also has been observed in lung cancer (Tanizaki et al., 2011; Ju et al., 2012). RET is reported to activate diverse intracellular signaling cascades that regulate cellular chemotaxis, branching morphogenesis, synaptic plasticity, survival, proliferation and migration, SH2B1 β adaptor protein perhaps takes part in the lung cancer's progression as a key enhancer of RET physiologic and pathologic activities. Moreover, the interaction of SH2B1 β with RET play an very important role in GDNF-induced neurite outgrowth that is essential for glioma's survival and differentiation (Zhang et al., 2006). SH2B1 interacts with the activation loop of TrkA and plays a specific role in TrkA-mediated differentiation in human neuroblastoma cells , as well as axonal survival in primary sympathetic neurons (Eggert et al., 2000). In addition, microinjection of the SH2 domain into transformed fibroblasts partially restored a normal actin stress fiber pattern suggesting a stimulatory role of SH2B1 in normal and malignant cell proliferation (Riedel et al., 2000). These studies suggested that SH2B1 plays a fundamental role in receptor tyrosine kinase-mediated cellular functions and physiologic and pathologic activities of many cancers.

Recently some domestic scholars found SH2B1 adaptor protein expression in colon and ovaries cancer were higher than neighboring normal tissues (Hua et al., 2010; Li et al., 2010). Our previous immunohistochemistry research also demonstrated that the positive cell rate and the score of staining intensity of SH2B1 in normal lung tissues, adjacent tissues of cancer tissues, and cancer tissues were gradually increased (Zhang et al., 2009), which probably participated in the cancer's development but its relevant mechanisms needed to further study. Therefore, in order to gain better insight into the clinical relevance of SH2B1 protein in NSCLC, the present study was carried out to investigate SH2B1 protein expression in a large number of archival NSCLC tissue samples and cell lines, and further to assess whether SH2B1 expression was correlated with clinicopathological parameters and prognosis in NSCLC patients.

Table 1. Clinicopathological Features of the Studied 114 Cases of NSCLC

Variables	Number of patients	Percentage (%)
Histology		
Squamous cell carcinoma	54	47.37
Adenocarcinoma	60	52.63
Age		
≤50	33	28.95
>50	81	71.05
Gender		
Male	82	71.93
Female	32	28.07
Smoking		
Yes	64	56.14
No	50	43.86
Grade(G)		
Well differentiated,G1	66	57.89
Moderately differentiated,G2	29	25.44
Poorly differentiated,G3	19	16.67
Tumor size(T)		
T1	17	14.91
T2	37	32.46
T3	42	36.84
T4	18	15.79
Clinical stage		
I	33	28.95
II	29	25.44
III	52	45.61
Lymph node metastases(N)		
Absent,N0	58	50.88
Hilar,N1	21	18.42
Mediastinal,N2	35	30.7
Recurrence*		
Yes	81	75
No	27	25

Note: *Six patients lost to follow-up because of telephone number changes or home moving

Materials and Methods

Patients and tissue preparation

In this retrospective study, we enrolled a total of 114 NSCLC patients who underwent surgical resection at the Department of Cardiothoracic Surgery of Xiangya Hospital in Central South University from September 2003 to March 2005. All patients had no history of previous malignancies, no history of radiotherapy or chemotherapy. Recurrence and metastasis were diagnosed by imaging evaluation, clinical examination, operation and postoperative pathological examination.

The main clinical and pathological variables of the patients were recorded in detail in Table 1.Eighty-two patients were men and 32 women, with a mean age of 55.31 years (range: 27–81 years, SD = 10.32). According to the 2009 TNM classification of malignant tumors by the International Union Against Cancer (UICC) and American Joint Committee on Cancer (AJCC), 54 cases were squamous cell carcinoma, 60 were adenocarcinoma. There were 33 cases in stage I (T1N0M0 13 cases, T2aN0M0 20 cases), 29 cases in stage II (T1N1M0 2 cases, T2bN0M0 5 cases, T2N1M0 5 cases, T3N0M0 17 cases), 52 cases in stage III (T1N2M0 2 cases, T2N2M0 7 cases, T3N1M0 8 cases, T3N2M0 17 cases, T4N0M0 3 cases, T4N1M0 6 cases, T4N2M0 9 cases). Considering pathological grading, 66 were staged as well differentiated (G1), 29 as moderately differentiated (G2), 19 as poorly differentiated (G3). Fifty-six patients with lymph node metastases were validated by conventional postoperative pathological examinations. Eighty-one patients experienced tumor recurrence after surgery.

15 cases of NSCLC and paired adjacent tissues were randomly collected from each patient during operation from January 2011 to August 2011, all of which were validated by pathologists. All specimens were snap-frozen immediately and stored in liquid nitrogen for RNA and total protein extraction. Before surgery informed consents were obtained from all patients, whose specimens were handled and made anonymous according to the ethical and legal standards, which was approved by the Research Ethics Committee of Central South University, Changsha, China.

Immunohistochemistry

Immunohistochemical staining was performed using the PV-6001 Two-Step IHC Detection Reagent following the manufacturer's recommended protocol (ZhongShan Goldenbridge Bio, Beijing, China). Briefly, antigen retrieval was carried out in 10 mmol/l citrate buffer (pH 6.0) for 15 min at 100°C in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature. Slides were incubated with SH2B1 goat polyclonal antibody (sc-8895, dilution 1:200) (Santa Cruz, CA, USA) at 4 °C overnight, followed by addition of HRP-labeled rabbit anti-goat polymers. Immunoreactive proteins were visualized with3', 3'-diaminobenzidine and counterstained with Mayer's hematoxylin. Negative control slides were probed with PBS under the same experimental conditions. Sections were blindly evaluated and scored by two independent board-certified pathologists (Liyuan Feng and Xueping Feng). Staining intensity and immunopositive cells of interest related classification standards as follows: A staining index (values 0–9), obtained as a product of staining intensity (0–3: 0 point = no intensity; 1 point = weak intensity; 2 points = moderate intensity; 3 points = strong intensity) and calculated proportion of immunopositive cells of interest (≤25% = 1, 25%–50% = 2, ≥ 50% = 3). Tumors were categorized into three groups according to the final staining index: negative or weak expression (scored 0–2), moderate expression (scored 3–4) and strong expression (5–9). NSCLC patients were dichotomized into low expression group (negative, weak, or moderate staining: 0–4) and high expression group (strong staining: 5–9) in order to better analyze the prognosis between groups.

Cell culture

NCI-H1395, LTEP-a-2, SPC-A-1 purchased from the chinese academy of sciences cells library, shanghai, China. NCI-H1395 was established from a stage II lung adenocarcinoma patient who was a 55 years female smoker. LTEP-a-2 was established from a poorly differentiated adenocarcinoma patient, SPC-A-1 was established from a pleura node metastasis from a poorly differentiated adenocarcinoma patient, which were all highly metastatic cell lines capable of generating high incidences of lymph node and lung metastasis. NCI-H520, A549 and the normal human bronchial epithelial (HBE) cell line purchased from Central Laboratory of Xiangya Medical School, Central South University, Changsha, China. The NCI-H520 cell line was derived by A.F. Gazdar and associates in 1982 from a sample of a lung mass taken from a male patient with squamous cell carcimoma of the lung. A549 cell line was initiated in 1972 by DJ. Giard, et al, through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. All cell lines were maintained as monolayer cultures in Roswell Park Memorial Institute (RPMI) Medium 1640 supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

Immunofluorescence

When on the exponential phase, 5 NSCLC cell lines and HBE cell line were separately adjusted 1×10^3/mL cell suspension by RPMI Medium 1640, then taken 2 drops cell suspension to prepare for cells grown on coverslips. After cultivation for 24 h , the cells were then rapidly fixed in 4% paraformaldehyde, perforated with 0.5% Triton for 15 minutes, blocked with 1% Bovine Serum Albumin (BSA) for 30 minutes, then we added goat poly-antibody against SH2B1 (sc-8895, dilution 1:200) (Santa Cruz, CA, USA) diluted with 1% BSA that hybridized overnight in 4 °C. After washing, we added rabbit anti-goat Cy3 antibody (wuhan Boster Bio, China; dilution 1:200) diluted with 1% BSA that hybridized 1 h in 37 °C, at last mounted by antifade solution then visualized using confocal microscopy on a Zeiss LSM 510. Each test, we seted the positive contrast, the negative contrast and fluorescent landmarks contrast .

Total RNA isolation and RT–PCR

Total RNA of tissue specimens and the 5 NSCLC cell lines and HBE cell line were isolated by TRIzol extraction liquid (Invitrogen, USA). Total RNA (1 μg) was reverse transcribed by High-Capacity cDNA Reverse Transcription Kits (Invitrogen, USA) according to manufacturer's instructions. Primers were designed and synthesized by Sangon Biological Engineering Technology and Services Co. Ltd (Shanghai, China). The primers for SH2B1 and β-action (internal control) were designed as follows: SH2B1 primer (304bp), forward 5'-GGAGATTTCGCCACATGACCT -3', reverse 5'-GGAGTTGCTGTTTCCACCTAAGAC -3'; β-action primer (361bp), forward 5'-TCCTTCCTGGGCATGGAGTC-3', reverse 5'-GTAACGCAACTAAGTCATAGTC-3'. RT product (1 μl) was amplified by PCR using the following conditions: 94 °C for 2 min; then 35 cycles for SH2B1 primers at 94 °C for 30 s, 59.5 °C for 30 s, and 72 °C for 1 min and 30 cycles for β-action primers at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min; and an extension at 72 °C for 5 min. PCR product (5 μl) was then electrophoresed on 1.5% agarose gel, and the intensity of bands was quantified by Image J (CA ,USA).

SH2B1 mRNA expression was determined as relative intensity of the PCR product bands from target sequences relative to the intensity of β-action mRNA. PCR experiments were repeated three times.

Western blotting

Harvest total protein lysates from tissue specimens and cell lines were all quantified by bicinchoninic acid protein assay kit (Beyotime, China). Total protein (40 μg) was separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blotting membranes were incubated with goat poly-antibody against SH2B1 (sc-8895, dilution 1:400) (Santa Cruz, CA, USA) at 4 °C overnight. After washing, they were incubated with HRP-labeled rabbit anti-goat IgG for 1.5 h at room temperature. Bands were visualized by employing the BeyoECL Plus Detection System (Beyotime, China). SH2B1 protein expression levels were quantified by Bio Rad -Image Lab Software (USA, CA) and represented as the densitometric ratio of the targeted protein to the housekeeping protein of GAPDH (Beyotime, China). The experiments were repeated three times.

Follow-up

Follow-up rate after surgery was 94.7% (108/114), 6 patients lost to follow- up because of telephone number changes or home moving. Recurrence and metastasis were diagnosed by imaging evaluation, clinical examination, operation, and postoperation pathological studies. Disease-free survival and Overall survival were calculated from the day of surgery to the date of tumor relapse or that of death. Deaths from other causes were treated as censored cases. Follow-up time was 5 years for each patient ranging from 2 to 60 months (mean = 40.90 months, SD = 21.046).

Statistical analysis

Continuous variables were expressed as mean ± SD. SH2B1 expression between cancer and paired adjacent tissues were compared by Student's two-tailed t-test. The protein expression and clinicopathological parameters were compared by the x^2 test. Survival analysis was undertaken using the Kaplan–Meier method and curves that were compared by the log-rank test. The Cox's proportional hazards model was used in the multivariate analysis to identify which factors were independent indicators for prognosis. All statistical analyses were performed with SPSS 18.0 software. A P-value <0.05 was considered to be statistically significant.

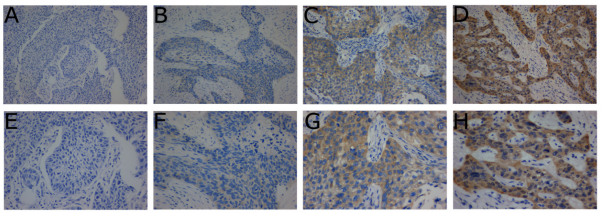

Figure 1. Immunohistochemistry of SH2B1 in Human NSCLC Tissues. A, E Negative control of SH2B1 in NSCLC tumor tissues (primary antibody replaced with PBS). B, F Weak expression of SH2B1 in NSCLC tumor tissues (Scored 1). C, G Moderate expression of SH2B1 in NSCLC tumor specimens (Scored 4). D, H Strong expression of SH2B1 in NSCLC tumor specimens (Scored 9) (original magnification×100 in A–D; ×200 in E–H)

Table 2. Correlations Between SH2B1 Expression and Clinicopathological Variables in Patients with NSCLC

Variables	Total	SH2B1 expression 0-2	3-4	5-9	χ^2	P-value*
Histology						
Squamous cell carcinoma	54	12	11	37	4.344	0.115
Adenocarcinoma	60	10	19	25		
Age						
≤50	33	5	7	21	1.604	0.448
>50	81	17	23	41		
Gender						
Male	82	17	24	41	2.312	0.315
Female	32	5	6	21		
Smoking						
Yes	64	12	22	30	5.138	0.077
No	50	10	8	32		
Grade(G)						
G1	66	18	15	33	6.487	**0.039**
G2+G3	48	4	15	29		
Tumor size(T)						
T1 +T2	54	16	12	26	7.062	**0.029**
T3 +T4	60	6	18	36		
Clinical stage						
I -II	62	18	14	30	8.294	**0.016**
III	52	4	16	32		
Lymph node metastases(N)						
N0	58	17	15	26	8.126	**0.017**
N1+N2	56	5	15	36		
Recurrence**						
Yes	81	7	22	52	10.98	**0.004**
No	27	9	8	10		

Note: *P< 0.05 was considered to be statistically significant; **Six patients lost to follow-up because of telephone number changes or home moving

Table 3. Multivariate Cox Model Analysis of Disease-free Survival and Overall Survival

Variable	Disease-free Survival		Overall Survival	
	Hazard ratio (95% CI)	P-value	Hazard ratio(95% CI)	P-value
Histology (Adenocarcinoma/Squamous cell carcinoma)	0.968(0.580-1.614)	0.899	0.798(0.446-1.429)	0.448
Age (≤50/>50)	0.638(0.371-1.096)	0.103	0.779(0.427-1.423)	0.417
Gender (Female/Male)	1.175(0.571-2.418)	0.661	1.373(0.598-3.157)	0.455
Smoking (Yes/No)	1.373(0.749-2.517)	0.306	1.406(0.695-2.844)	0.343
Grade (G) (G2+G3/G1)	1.179(0.697-1.993)	0.539	1.288(0.704-2.357)	0.411
Tumor size (T) (T3 +T4/ T1 +T2)	1.887(0.990-3.596)	0.054	1.927(0.913-4.067)	0.085
Clinical stage (III/ I +II)	4.445(1.361-14.519)	**0.014**	3.624(1.029-12.766)	**0.045**
Lymph node metastases (N) (-/+)	1.119(0.382-3.276)	0.838	1.011(0.313-3.271)	0.985
SH2B1expression (High/Low)	2.596(1.569-4.296)	**0.000**	2.544(1.403-4.612)	**0.002**

Note: All the clinicopathological variables listed in table were included in the multivariate analysis; Abbreviation: 95% CI, 95% confidence interval; P-values in bold were statistically significant

Results

Immunohistochemistry results and correlations between SH2B1 expression levels and clinicopathological variables

A total of 114 archival NSCLC tumor samples with intact clinicopathological data were initially identified for SH2B1 protein expression by immunohistochemistry and correlated with clinicopathological parameters. Among all the samples analyzed, 62 (54.39%) cases demonstrated strong SH2B1 protein expression (Scored 5–9; Figure 1D, H), 30 (26.31%) cases with moderate SH2B1 protein expression (Scored 3–4; Figure 1C, G) and 22 (19.30%) cases with negative or weak expression (Scored 0–2; Figure 1B, F). Positive SH2B1 immunostaining was predominantly diffusely distributed throughout the cytoplasm of NSCLC tumor cells (Figure 1). Moreover, the x^2 test was applied to assess the association between SH2B1 protein level and different clinicopathological variables (Table 2). As a result, SH2B1 overexpression was significantly associated with NSCLC tumor size (P = 0.029), tumor grade (P = 0.039), clinical stage (P = 0.016), lymph node metastasis (P = 0.017) and recurrence (P = 0.004), respectively. While there was no significant relationship between SH2B1 protein level and variables such as tumor histology, age, gender, smoking history (all P > 0.05).

SH2B1 mRNA and protein expression in NSCLC tissues

15 paired freshly collected clinical NSCLC tissue specimens were further investigated by RT–PCR and Western blotting in order to make up the lack of adjacent tissues as a control and better gain SH2B1 mRNA expression pattern . RT–PCR results using the SH2B1 specific primers indicated that SH2B1 mRNA was readily detectable in all the 15 NSCLC tissues and in 15 paired adjacent tissues, while the levels of SH2B1 mRNA expression levels in NSCLC specimens were significantly higher than that in the corresponding adjacent tissues (0.81 ± 0.17 vs. 0.41 ± 0.14; P = 0.002; Figure 2A , C). Consistent with the mRNA material, the SH2B1 protein was also detectable in all the 15 NSCLC tissues and in 15 paired adjacent tissues, the levels of SH2B1 protein expression in NSCLC carcinomas were significantly elevated with respect to the corresponding adjacent samples, too (0.68 ± 0.21 vs. 0.43 ± 0.29; P = 0.019; Figure 2B, C).

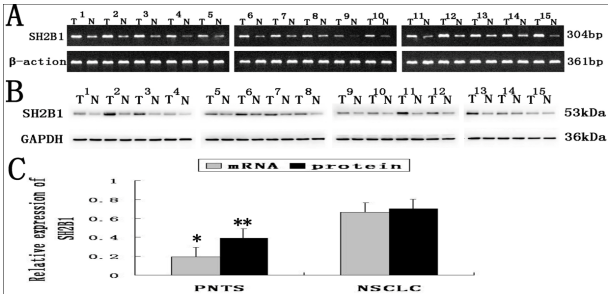

Figure 2. Expression of SH2B1 mRNA and Protein in 15 Paired Human NSCLC and Adjacent Noncarcinoma Tissues. The abundance of SH2B1 mRNA and protein was shown relative to the levels of β-actin mRNA or GAPDH protein, respectively. A: Reverse transcriptase-polymerase chain reaction results. B: Western blotting results. C: Relative expression levels of SH2B1 mRNA and protein in tissue specimens. The Student's t test demonstrated that expression levels of SH2B1 mRNA and protein in NSCLC tissues were significantly higher than those in PNTS. The single asterisk and double asterisks indicated that both SH2B1 mRNA and protein levels in NSCLC tissues were significantly higher than that in PNTS (P = 0.002, P = 0.019) respectively. T tumor sample, N noncarcinoma tissues, PNTS paired noncarcinoma tissues

Expression of SH2B1 mRNA and protein in NSCLC cell lines

Furthermore, the expression pattern of SH2B1 in the cultured human NSCLC cell lines was also investigated by Immunofluorescence, RT–PCR and Western blotting. SH2B1 protein expression was detectable in all cell lines by Immunofluorescence, but the expression of all the five NSCLC cell lines were significantly higher than that in the normal HBE cell line (Figure 3). As can be seen that both mRNA and protein expression of SH2B1 were detected in all the five NSCLC cell lines and the normal HBE cell line (Figure 4A, B). More importantly, compared to the normal HBE cell line, SPC-A-1 and LTEP-a-2 with high metastatic ability revealed the highest SH2B1 protein expression, whereas the other three poorly metastatic NSCLC cell lines (NCI-H520, A549, NCI-H1395) with a relative low SH2B1 protein expression, which were consistent with their low metastatic ability in nude mouse xenotransplanted model.

The comparatively quantitative SH2B1 mRNA and protein level was calculated by us that the levels of SH2B1 protein were disproportionate with its mRNA expression levels in these cell lines (Figure 4C).

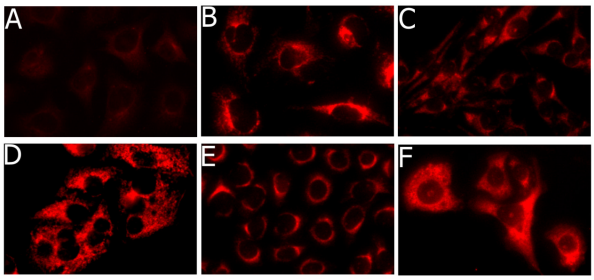

Figure 3. Expression of SH2B1 in the Five Selected Human NSCLC Cell Lines and Normal HBE Cell Line was Applied by Cy3 Antibody Immunofluorescence Dyeing Method, Which Major Manifested in the Cell Plasma. SH2B1 protein expression was detectable in all cell lines by Immunofluorescence, but the expression of all the five NSCLC cell lines were significantly higher than that in the normal HBE cell line (original magnification×400 in A–F). (A: HBE; B: NCI-H520; C: A549; D: NCI-H1395; E: SPC-A-1; F: LTEP-a-2)

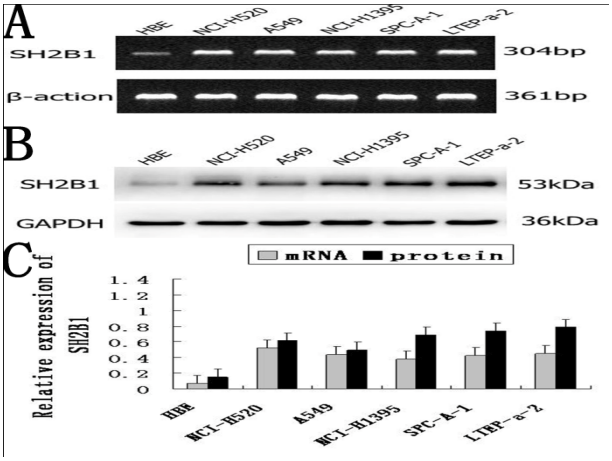

Figure 4. Expression of SH2B1 mRNA and Protein in the Five Selected Human NSCLC Cell Lines and HBE Cell Line. The abundance of of SH2B1 mRNA and protein was shown relative to the levels of β-actin mRNA or GAPDH protein. A: Representative reverse transcriptase-polymerase chain reaction results. B: Representative Western blotting results. C: Relative expression levels of SH2B1 mRNA and protein

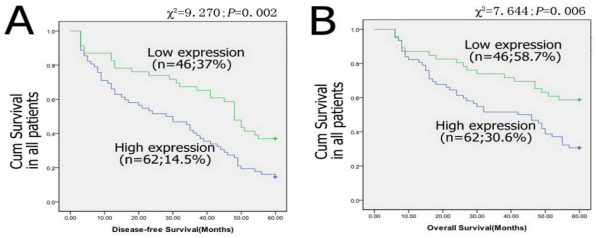

Figure 5. Kaplan-Meier Survival Analysis of Disease-Free Survival (A) and Overall Survival (B) in All Patients were Grouped According to Low and High SH2B1 Expression. The log-rank test was applied to calculate P-value

Correlation between SH2B1 protein expression and patients' survival analysis

108 patients with NSCLC followed-up (6 patients lost) were subdivided into two groups according to the SH2B1 protein expression levels: 48 patients in the low expression group and 60 patients in the high expression group. The overall survival rate and overall disease-free survival rate in the 108 patients was 42.6 and 24.1%. Patients in the high expression group had both poorer disease-free and poorer overall survival than that in the low expression group by using the Kaplan–Meier analysis (37 vs. 14.5%, Figure 5A; 58.7 vs. 30.6%, Figure 5B; both $P < 0.05$). In the multivariate Cox regression analysis, patients' tumor histology, age, gender, smoking history, tumor size, grade, clinical stage, lymph node metastasis and SH2B1 expression were included as important clinicopathological parameters.These results revealed that SH2B1 protein expression had a significant correlation with NSCLC prognosis and was found to be an independent prognostic factor of outcomes in patients with NSCLC after tumour resection (hazard ratio =2.544, 95% confidence interval (CI) = 1.403–4.612, P = 0.002) (Table 3). Consequently, SH2B1 protein expression pattern may be a valuable prognostic marker for NSCLC patients.

Discussion

Previous many disquisitive analysis have revealed the presence of SH2B1 transcripts and protein in heart, brain, lung, skeletal muscle, testis, pancreas, adipose tissue (Osborne et al., 1995; Rui et al., 1997), and stable overexpression of SH2B1β in PC12 cells (Wang et al., 2004). In our present study, we further proved that the protein expression level of SH2B1 was significantly higher in NSCLC specimen than that in the corresponding adjacent tissues, which was consistent with several other reports (Hua et al., 2010; Li et al., 2010). Mean-while, SH2B1 protein was obviously overexpressed in all the 5 NSCLC cell lines compared with the normal HBE cell line. However, the underlying mechanisms for the high expression of SH2B1 protein in tumor remain incompletely understood but likely involved in the tyrosine kinase transduce signaling. It can be seen that the family of SH2 adaptor proteins usually take part in a variety of human cancers' pathological processes because of the enhancement of tyrosine kinase's activation which contributed to the activation of many proto-oncogenes and the abnormity of signal transduction, for example, Grb2 overexpresses in breast cancer (Pawson et al., 1997), the sequence missing of Src C terminal interacting with SH2 structure domain induces colon cancer (Zhou et al., 2004), the Ras-GAP mutation enhances SH2 structure domain proteins' activities in basal cell carcinoma (Firedman et al., 1993). SH2B1 adaptor protein binds of its SH2 domain to a phosphorylated tyrosine in the tyrosine kinase JAK2, overexpression of SH2B1 dramatically stimulates the JAK2 activity through JAK/STAT signaling pathway, resulting in a significant increase of JAK2 phosphorylation and multiple other cellular proteins which contribute to the activation of many proto-oncogenes that lead to a host of physiological and pathological problems, including cancer (Lacronique et al., 1997).

SH2B1 protein expression was detectable in all cell lines by Immunofluorescence, but the expression of all the five NSCLC cell lines were significantly higher than that in the normal HBE cell line. Compared to the normal HBE cell line and the poorly metastatic NSCLC cell lines, SPC-A-1 and LTEP-a-2 with the highest metastatic ability

in vivo displayed the strongest SH2B1 protein expression. These data revealed a potential association between SH2B1 overexpression and malignant phenotype such as metastasis, which was consistent with other reports in colon cancer cell lines (Huang et al., 2010; Tang et al., 2011), in which highly aggressive or metastatic cancer cell lines overexpressed SH2B1. SH2B1β shuttles continuously between the cytoplasm and nucleus (Chen et al., 2004), which also functions as an adapter or scaffolding protein that recruits Rac and perhaps other proteins to activate membrane receptor-JAK complexes or receptor tyrosine kinases where they are then positioned appropriately to regulate the actin cytoskeleton and promote membrane ruffling and cell motility (Diakonova et al., 2002). This cell migration was critical for tumor formation and metastasis. Further deleting or mutating the SH2 domain of SH2B1β inhibited changes in the actin cytoskeleton, resulting in decreased cell movement, lamellipodia activity and membrane ruffling. Therefore, SH2B1βplayed an important role in cell motility and may promote lung tumorigenesis and metastasis. It is widely accepted that metastasis contribute to the high mortality rate for patients with NSCLC. Our data revealed that high SH2B1 protein expression was found to well correlate with lymph node metastasis and predicted worse prognosis. Although close association between SH2B1 expression and NSCLC metastasis has been established in our study, the possible mechanisms are still unclear that needed further investigations. Recent some studies have suggested that SH2B1 participate in tumor metastasis through its regulation of processes intrinsic to tumor cells and the surrounding tumor microenvironment depending on the interaction with multiple ligand-activated receptor tyrosine kinases, PDGF , IGF-I , FGFR3 , leptin and so on (Tsuchiya et al., 1999; Riedel et al., 2000; Yousaf et al., 2001; Kong et al., 2002; Somasundar et al., 2004), which may be a good illumination to tumor metastasis. In addition, the results of 108 archival NSCLC patients investigated by immunohistochemistry revealed that high SH2B1 expression level was closely associated with tumor grade, tumor size, clinical stage, lymph node metastasis and recurrence, which indicated the importance of SH2B1 in NSCLC progression and metastasis after surgical resection.

Finally, patients with high SH2B1 expression were significantly associated with shorter disease-free and overall survival by using the Kaplan–Meier analysis. Therefore, SH2B1 protein expression pattern might be a valuable prognostic marker for NSCLC patients. More importantly, we educed SH2B1 expression level was an independent prognostic indicator for patients with NSCLC in Cox multivariate analysis including 9 clinicopathological parameters, such as patients' histology, age, gender, smoking, grade, tumor size, clinical stage, lymph node metastasis and SH2B1 expression level.

In summary, our present study demonstrated that elevated SH2B1 expression levels was associated with the progression and poor prognosis in patients with NSCLC, which indicates that SH2B1 may serve as a valuable prognosis marker in NSCLC. However, the possible underlying mechanisms for its participation in tumor progression are still unclear; therefore, next step we will make a further research from cell signaling pathway in order to gain a better molecular mechanism understanding in this field.

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