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Original Article

Elevated expression of SHIP2 correlates with poor prognosis in non-small cell lung cancer

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Abstract: SH2-containing inositol 5'-phosphatase 2 (SHIP2) is a vital regulator of phosphoinositide pools in metabolic pathways and is considered to downregulate phosphatidylinositol 3'-kinase signaling, which underlies the development of several kinds of human cancers. However, SHIP2 expression in non-small cell lung cancer (NSCLC) and its relationship with the clinical characteristics of NSCLC remain poorly understood. In this study, one-step quantitative reverse transcription-polymerase chain reaction and immunohistochemistry analysis with tissue microarray was used to evaluate SHIP2 expression in NSCLC and to investigate the relationship of this expression to NSCLC prognosis. Results showed that the expression of SHIP2 messenger RNA and protein was significantly higher in NSCLC than in corresponding non-cancerous tissues (both $p < 0.05$). SHIP2 protein expression in NSCLC was related to lymph node metastasis ($p = 0.042$), TNM stage ($p = 0.036$), and 5-year survival rate ($p = 0.046$). The Kaplan-Meier method and log-rank test suggested that high SHIP2 expression, tobacco consumption, and advanced tumor stage were significantly associated with low survival of NSCLC patients. The results of this research suggested that SHIP2 expression was correlated with malignant phenotypes of NSCLC and may thus serve as a poor prognostic factor and valuable oncogene for NSCLC.

Keywords: SHIP2, NSCLC, qPCR, immunohistochemistry, prognosis

Introduction

Lung cancer (LC) is one of the most common and lethal human malignancies worldwide [1, 2]. In China, lung cancer mortality has been increasing by more than 400% over the past three decades and is the second leading cause of death [3]. LC is generally composed of small-cell (SC) and non-small-cell lung cancer (NSCLC). The latter accounts for approximately 85% of all LC cases, with adenocarcinoma (AD) accounting for 40% and squamous cell carcinoma (SQ) for 35% [4, 5]. Patients with early-stage NSCLC have relatively high long-term survival rates after surgery. However, the majority of NSCLC patients accounting for 80% are diagnosed in advanced stages. Despite vital improvements in diagnostic and therapeutic techniques for NSCLC, prognosis for patients remains poor, with a low 5-year survival rate of 15% [5, 6]. At present, researchers are focusing on the potential role of certain biological factors in carcinogenesis as prognostic markers in NSCLC [7, 8]. The identification of novel and valuable molecular prognostic factors is highly significant in facilitating the choice of potential therapeutic targets for NSCLC treatment.

SH2-containing inositol 5'-phosphatase 2 (SHIP2) is an important regulator of phosphoinositide pools in metabolic pathways [9] and is considered to downregulate phosphatidylinositol 3'-kinase (PI3K) signaling, which underlies the development of several kinds of human cancers [10-12]. As a result, a number of PI3K pathway components are being targeted for anticancer drug discovery [13]. So far, a growing number of studies have indicated the possible functions of SHIP2 in cancer-relevant pathways, such as the regulation of cell adhe-

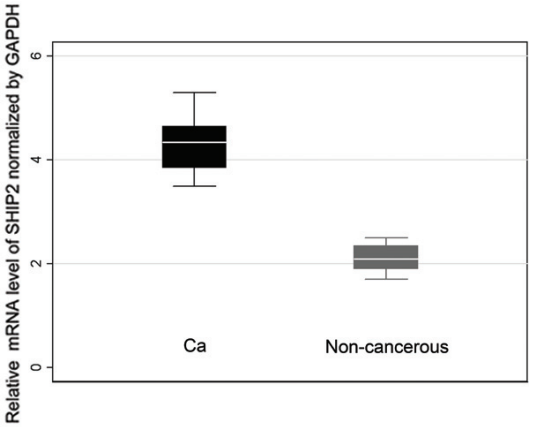

Figure 1. Expression of SHIP2 mRNA in non-small cell lung cancer (NSCLC) and tumor adjacent tissue. One-step quantitative real-time polymerase chain reaction (qPCR) was employed to evaluate SHIP2 mRNA expression levels in NSCLC (cancer) compared with tumor adjacent tissue (normal). Normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels, the SHIP2 mRNA level in NSCLC tissue is significantly higher than that in corresponding non-cancerous tissue ($p < 0.05$). Error bar is standard error.

sion downstream of Src tyrosine kinases [14] and of epidermal growth factor receptor internalization and degradation [15]. Two recent studies have reported that SHIP2 exhibits oncogenic characteristics, and that high SHIP2 expression indicates unfavorable prognosis in breast cancer and laryngeal squamous cell carcinoma (LSCC) [16, 17]. These findings suggest that SHIP2 plays critical roles in tumorigenesis. Therefore, SHIP2 may be a candidate biomarker for prognosis prediction in certain human cancers. However, the relationship of SHIP2 expression with its substantial clinicopathological role in NSCLC has not yet been elucidated.

In the present research, we explored SHIP2 gene and protein expression in several NSCLC and matched tumor-adjacent normal tissues. Moreover, the association of SHIP2 expression with clinicopathological features of NSCLC was analyzed. Finally, the prognostic significance of SHIP2 protein expression in NSCLC was investigated.

Materials and methods

NSCLC patients and tissue microarray (TMA) analysis

Formalin-fixed, paraffin-embedded tumor samples from 100 NSCLC cases (45 SQ and 55 AD) and matched peritumoral tissue specimens were collected from NSCLC patients who underwent surgery at the Affiliated Hospital of Nantong University from 2005 to 2006. The mean age of patients at the time of surgery was 62.6 years (range = 35 years to 81 years). Related clinical data were collected, including gender, age, tumor size, histologic type, smoking status, tumor differentiation, TNM stage, lymph node metastasis, and 5-year follow-up survival. None of the patients received radiotherapy, chemotherapy, or immunotherapy prior to lobectomy. A written informed consent and any related picture were acquired from each patient for publication of this study. The study protocol was approved by the Human Research Ethics Committee of each hospital.

Representative 2.0 mm tissue core samples from each patient were used for TMA analysis (Shanghai Outdo Biotech, Shanghai, China).

Quantitative real-time polymerase chain reaction (qPCR) analysis

Freshly frozen NSCLC tumors (eight SQ and eight AD) and adjacent peritumoral tissue samples were obtained from the Kunshan First People's Hospital Affiliated to Jiangsu University (Suzhou, China). Total RNA extraction, quality control, and one-step qRT-PCR analysis were performed as previously described [18]. The primers for SHIP2 were as follows: forward primer 5'-TCG TCA CCA GCG ACC ATT C-3' and reverse primer 5'-AGC CCT TTC TTG GAG ATG AAC TG-3'. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was used to standardize the measurements of the target gene using the following primers for GAPDH: forward primer 5'-TGC ACC ACC AAC TGC TTA GC-3' and reverse primer 3'-GGC ATG GAC TGT GGT CAT GAG-5'. Amplification conditions consisted of 30 min at 42 °C for reverse transcription and 2 min at 94 °C for Taq activation, followed by 35 cycles at 95 °C for 20 s, 56 °C for 20 s, and elongation at 72 °C for 30 s. Each measurement was performed in triplicate.

Immunohistochemistry (IHC) analysis

IHC analysis was performed as previously described [18]. Deparaffinized sections (4 μm thick) from array blocks were separately stained on an Autostainer Universal Staining System (LabVision, Kalamazoo, MI, USA) using polyclonal goat anti-SHIP2 antibody (Santa Cruz

Table 1. Correlation of high SHIP2 expression with clinicopathological characteristics in NSCLC

Groups	No.	SHIP2		χ^2	*p* value
		+	%		
All patients	100	55	55.0		
Gender					
Male	68	37	54.4	0.0297	0.863
Female	32	18	56.3		
Age (years)					
≤ 60	38	19	50.0	0.6191	0.431
> 60	62	36	58.1		
Tumor diameter (cm)					
≤ 3	18	10	55.6	0.0027	0.958
> 3	82	45	54.9		
Histologic type					
Squamous cell carcinoma	45	26	57.8	0.2551	0.614
Adenocarcinoma	55	29	52.7		
Smoking status					
Tobacco consumption	57	32	56.1	0.0696	0.792
No tobacco consumption	43	23	53.5		
Tumor differentiation					
Well	7	5	71.4	1.0057	0.605
Moderate	65	34	52.3		
Poor	28	16	57.1		
Lymph node metastasis					
Yes	49	32	65.3	4.1233	0.042*
No	51	23	45.1		
TNM stage					
Stage I	48	20	41.7	6.6397	0.036*
Stage II	27	18	66.7		
Stage III and IV	25	17	68.0		
Five years' survival					
Yes	34	14	41.2	3.9774	0.046*
No	66	41	62.1		

*$p < 0.05$.

Biotechnology, Santa Cruz, CA, USA). The secondary antibody used was horseradish peroxidase-conjugated anti-goat antibody (Dako Cytomation, Carpinteria, CA, USA). For negative controls, phosphate-buffered saline was used instead of the primary antibody. Blind SHIP2 immunostaining evaluations and independent observation were simultaneously performed. IHC results were analyzed according to a previously described method [19, 20]. In a typical procedure, the immunohistochemistry score (IHS) was determined by evaluating both staining intensity and density. The staining intensity was scored as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The percentage of SHIP2-positive cells was also scored according to four categories: 1 was given for 0% to 10%, 2 for 11% to 50%, 3 for 51% to 80%, and 4 for 81% to 100%. The sum of the intensity and percentage scores gave the final IHS. Samples with a total score below 3 (IHS ≤ 3) were considered to have low SHIP2 expression, whereas those with a total score above 4 (IHS ≥ 4) were considered to exhibit high SHIP2 expression.

Statistical analysis

The SHIP2 mRNA expression in fresh-frozen NSCLC compared with matching peritumoral tissue normalized to GAPDH was analyzed with the Wilcoxon non-parametric signed-rank test. The associations between clinicopathologic variables and SHIP2 protein expression were examined with χ^2 tests. Survival curves were calculated using the Kaplan-Meier method. Factors shown to be of prognostic significance with the univariate Cox regression model were subsequently investigated with the multivariate Cox regression model. For all tests, the significance level for statistical analysis was set at $p < 0.05$. Data were analyzed using STATA Version 12.0 (Stata Corporation, College Station, TX).

Results

Evaluation of SHIP2 mRNA expression by qPCR

Total RNA was extracted from 16 NSCLC tissues and then subjected to one-step qPCR to determine SHIP2 mRNA expression. We also analyzed samples from the matched tumor adjacent tissues to compare mRNA expression in non-cancerous tissue. By normalizing to GAPDH, the means of SHIP2 mRNA in NSCLC and of the corresponding non-cancerous tissue were determined as 4.34 ± 0.133 and 2.12 ± 0.064, respectively ($p < 0.05$). SHIP2 expres-

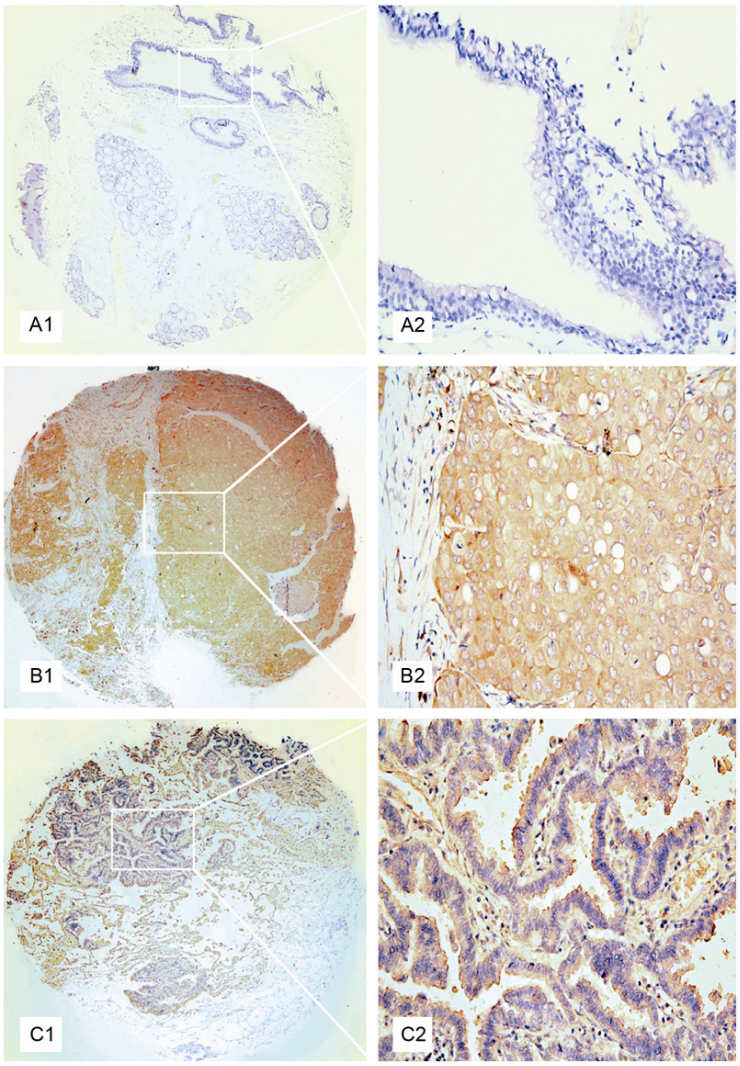

Figure 2. Representative pattern of SHIP2 protein expression in non-small cell lung cancer (NSCLC) and adjacent non-cancerous tissue. A1 and A2: Negative immunohistochemical (IHC) staining of SHIP2 in tumor adjacent non-cancerous tissue. B1 and B2: Positive IHC reaction for SHIP2 in lung squamous cell carcinoma (SQ) tissue. C1 and C2: Positive IHC reaction for SHIP2 in lung adenocarcinoma (AD) tissue. Original magnification ×40 in A1, B1, C1, ×400 in A2, B2, C2.

sion in the NSCLC samples was approximately 2.05-fold higher than that in matched non-cancerous tissues (**Figure 1**).

Association between SHIP2 expression and clinicopathological parameters of NSCLC

The association of high SHIP2 expression with the clinicopathological variables of 100 NSCLC patients is shown in **Table 1**. High SHIP2 expression was associated with lymph node metastasis ($p = 0.042$), TNM stage ($p = 0.036$), and 5-year survival rate ($p = 0.046$). By contrast, no significant association was found between SHIP2 expression and other clinical parameters, such as gender, age, tumor diameter, histologic type, smoking status, and tumor differentiation (**Table 1**).

SHIP2 expression in NSCLC by IHC

We performed IHC analysis with TMA to investigate SHIP2 expression in NSCLC. High SHIP2 expression was observed in 55 (55.0%) of the 100 NSCLC tumors compared with only 16 (16.0%) of the matched peritumoral lung tissue samples. Results showed statistical significance ($p < 0.001$) based on χ^2 test analysis and are consistent with the SHIP2 mRNA level found in fresh NSCLC samples. Positive staining was mainly localized in the cytoplasmic membrane and cytoplasm of NSCLC cells. Typically observed IHC staining modes for SHIP2 in NSCLC are sho wn in **Figure 2**.

Survival analysis

Based on univariate Cox regression analyses for all factors, high SHIP2 expression was a significant ($p = 0.004$) prognostic factor for NSCLC (**Table 2**). Smoking status ($p = 0.035$) and tumor TNM stage ($p = 0.047$) were also closely involved in patient survival. The multivariate Cox regression model further demonstrated that SHIP2 expression ($p = 0.001$), smoking status ($p = 0.009$), and tumor TNM stage ($p = 0.009$) were the strongest predictors of survival (**Table 2**). Kaplan-Meier survival curves showed that NSCLC patients with low SHIP2 expression, no tobacco consumption, and early TNM stage had a significantly favorable survival time (**Figure 3**).

Discussion

Phosphatidylinositol-3,4,5-trisphosphate (PIP3) is an important second messenger pro-

Table 2. Univariate and multivariable analysis of prognostic factors in NSCLC for 5-year survival

	Univariate analysis			Multivariable analysis		
	HR	p > |z|	95% CI	HR	p > |z|	95% CI
SHIP2 expression						
High versus Low	2.12	0.004*	1.265-3.547	2.39	0.001*	1.411-4.048
Gender						
Male versus Female	1.29	0.353	0.756-2.194			
Age						
> 60 years versus ≤ 60 years	1.64	0.058	0.984-2.746			
Tumour diameter						
> 3 cm versus ≤ 3 cm	1.21	0.576	0.618-2.380			
Histologic type						
Squamous cell carcinoma versus Adenocarcinoma	0.95	0.848	0.587-1.548			
Smoking status						
Tobacco consumption versus No tobacco consumption	1.72	0.035*	1.039-2.849	2.00	0.009*	1.191-3.342
Tumor differentiation						
Well versus Moderate versus Poor	1.38	0.143	0.896-2.132			
Lymph node metastasis						
Positive versus Negative	1.49	0.109	0.915-2.429			
TNM stage						
Stage I versus Stage II versus Stage III and IV	1.34	0.047*	1.004-1.798	1.50	0.009*	1.108-2.026

*$p < 0.05$.

duced by PI3K involved in the regulation various cell functions, including cellular survival, adhesion, and migration [21, 22]. Disruption of the PI3K pathway underlies cancer development. Therefore, certain components of this pathway can be targeted for anticancer therapy [23]. SHIP2 is a highly significant lipid phosphatase that acts downstream of PI3K and regulates multiple oncogenic signaling pathways by dephosphorylating PIP3 to produce phosphatidylinositol-3,4-bisphosphate. The potential pathways include the PI3K and Akt cascade [24], as well as the Ras and mitogen-activated protein kinase cascade [25]. A recent study has reported that high SHIP2 expression in breast cancer cells promotes cell proliferation, whereas targeted suppression of SHIP2 inhibits tumor growth and metastases in nude mice [23]. Moreover, elevated SHIP2 protein expression has been more frequently detected in clinical samples of breast cancer tissues than in non-cancerous breast tissue samples, thereby indicating the prognostic significance of SHIP2 as a biomarker for breast cancer [16]. These findings indicate that SHIP2 can be a novel anticancer target for cancer treatment strategies. Thus, we aimed to verify the relationship of SHIP2 expression with diverse clinicopathological parameters in NSCLC.

In the current research, the SHIP2 mRNA and protein levels in NSCLC and matched non-cancerous tissues were evaluated by qPCR and IHC, respectively. The qPCR and IHC results were consistent and showed that SHIP2 was highly expressed in NSCLC compared with that in adjacent non-cancerous tissues, suggesting that SHIP2 expression played an important role in NSCLC tumorigenesis. The expression pattern of SHIP2 protein, which is mainly localized in the cytoplasmic membrane and cytoplasm of NSCLC cells, is also similar to that previously observed in LSCC [17]. Moreover, we found that high SHIP2 expression in NSCLC was related to certain particular clinicopathological characteristics, including lymph node metastasis, TNM stage, and 5-year survival rate.

Univariate analysis results showed that apart from SHIP2 expression, the smoking status and TNM stage were also correlated with the lifespan of NSCLC patients. Multivariate analysis subsequently proved that high SHIP2 expression, tobacco consumption, and advanced TNM stage were independent factors for poor NSCLC prognosis in this research. The survival analysis data were also consistent with the aforementioned conclusion, suggesting that SHIP2 can serve as a prognostic marker in

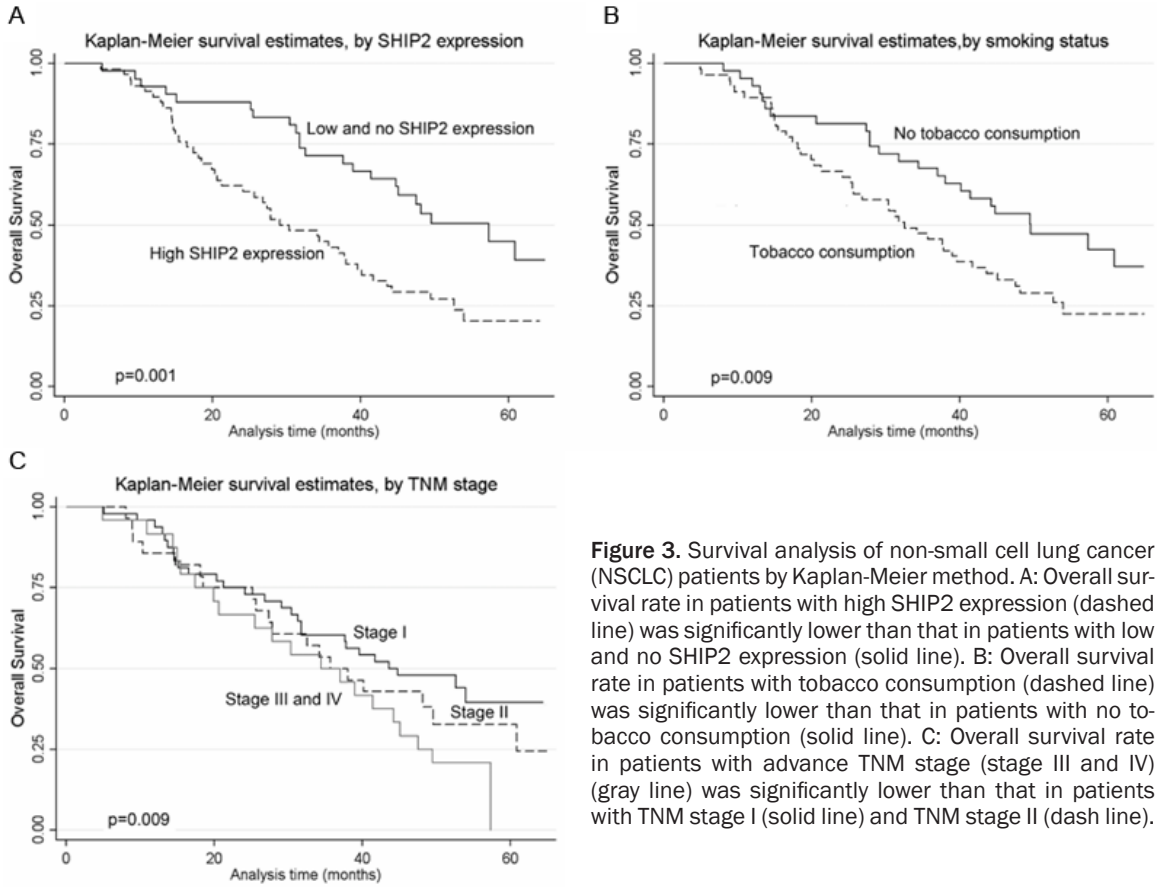

Figure 3. Survival analysis of non-small cell lung cancer (NSCLC) patients by Kaplan-Meier method. A: Overall survival rate in patients with high SHIP2 expression (dashed line) was significantly lower than that in patients with low and no SHIP2 expression (solid line). B: Overall survival rate in patients with tobacco consumption (dashed line) was significantly lower than that in patients with no tobacco consumption (solid line). C: Overall survival rate in patients with advance TNM stage (stage III and IV) (gray line) was significantly lower than that in patients with TNM stage I (solid line) and TNM stage II (dash line).

the HCC of our previous research (paper in press) and in LSCC [17].

Although the relationship between SHIP2 expression and prognosis has only been explored in breast cancer and LSCC to date [16, 17], the role of SHIP2 in carcinogenesis is attracting increased attention [26, 27]. SHIP2 clearly has a vital function in cancer development and progression. Our research team has been collecting different types of cancer tissue samples in addition to HCC and NSCLC, including intestinal cancer, pancreatic cancer, ovarian cancer, and nasopharyngeal cancer. In the future, we aim to explore further the relationship between SHIP2 expression and these various cancers.

In summary, this study was the first to report on the differential expression of SHIP2 in NSCLC. Our results indicated that SHIP2 can be used as a novel prognostic biomarker. Our findings indicated high SHIP2 expression in NSCLC tissue and that this high expression was associated with unfavorable prognosis. Further experiments must be conducted to demonstrate the potential mechanisms of SHIP2 involvement in NSCLC.

Disclosure of conflict of interest

None.

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