SFRP1 Decline in Non-Small Cell Lung Cancer (NSCLC) and its Importance in Prognoses

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Abstract

Background: In the present study, we sought to detect the expression of secreted frizzled-related protein-1 (*SFRP1*) and investigate its role in the progression and prognosis of patients with non-small cell lung cancer (NSCLC).

Methods: The expression of *SFRP1* at both mRNA and protein level were examined by quantitative real-time polymerase chain reaction (qRT-PCR) and Immunohistochemistry analysis, respectively. The relationship between *SFRP1* expression and clinical factors of patients with NSCLC was analyzed by chi-square test. Transwell assay was conducted to determine the influences of *SFRP1* on migratory and invasive of NSCLC cells. Kaplan-Meier analysis was used to describe the overall survival of NSCLC patients. Cox regression analysis was conducted to estimate the prognostic value of *SFRP1* in NSCLC.

Results: The expression of *SFRP1* was down-regulated in NSCLC tissues compared to that in adjacent normal controls both at mRNA and protein level ($P < 0.05$). And the low *SFRP1* expression was related to the distant metastasis, vascular invasion and TNM stage. The overexpression of *SFRP1* in vitro significantly inhibited the migration and invasion of NSCLC cells. The overall survival of patients with high *SFRP1* expression was proved to be longer than those with low expression (log rank test, $P < 0.001$). In addition, univariate and multivariate analyses suggested that *SFRP1* was an independent prognostic molecule marker for NSCLC patients.

Conclusion: Taken together, our findings indicated that the decreased of *SFRP1* could influence the cell migration and invasion as well as be regarded as an independent prognostic marker in NSCLC.

Background

Lung cancer is the most frequent cancer that causes the majority of cancer-related deaths all over the world [1]. It is categorized into several subtypes and non-small cell lung cancer (NSCLC) is the most common type, which accounts for about 80–85% of all lung cancers [2, 3]. NSCLC is a kind of molecularly heterogeneous malignancy with high proliferation rate, even patients with the same tumor node metastasis (TNM) stage appear varied clinical prognosis and outcomes [4, 5]. Standard therapies for NSCLC are mainly surgery, radiation therapy, and chemo therapy combined or alone [6, 7]. Recently, though great and efficient advancements and improvements have been made in treatment modalities, the prognosis of NSCLC patients remains poor [8, 9]. The five-year survival rate of NSCLC patients is limited to 15%, even the 5-year post-resection survival rate is only 20–30% [10]. Therefore, it is urgently need to discovery biomarkers that could be used as prognostic factors for NSCLC patients.

SFRP1 is a member of *SFRPs* family which are regarded as inhibitors of the Wnt signaling pathway that participate in multiple biological progressions such as embryogenesis and tumorigenes and is important for the maintenance of gut homeostasis and embryonic development [11–13]. It is located at chromosome 8p12-p11.1 and can encode a secreted glycoprotein with the molecular mass of 35 kDa [14–16]. *SFRP1* is a type of newly discovered candidate anti-oncogene and its aberrant expression has

been found in several cancers, such as glioma and prostate cancer and [17, 18]. However, it had mostly focused on the methylation of *SFRP1* in NSCLC according to previous studies [19, 20], the prognostic value of *SFRP1* was never reported.

In this study, we detected the expression of *SFRP1* in NSCLC patients and analyzed its relationship with clinical factors of patients with NSCLC. Besides, we estimated its influence on cell migration and invasion of NSCLC cells. What's more, the prognostic value of *SFRP1* for NSCLC patients was evaluated.

Methods And Materials

Patients and specimens

A total of 125 patients with pathologically diagnosed NSCLC who suffered from surgical resection Cangzhou Central Hospital were recruited in this study, including 59 males and 66 females. None of them had received any radio- or chemo- therapy before surgery. Besides, 51 out of 125 patients were randomly selected to resect the adjacent normal tissues which were taken as internal controls. This study was approved by the Ethic Committee of the hospital and all participants signed the written informed consents in advance.

Tumor tissues and adjacent normal tissues were collected from patients with NSCLC and frozen in liquid nitrogen immediately. Then the tissues samples were severally stored at -80°C for RNA extraction. Detailed information of patients including gender, smoking status, histology, vascular invasion, distant metastasis, and TNM stage were summarized in a database. A 5-years' follow-up was conducted with all patients and those who were died from unexpected events or other diseases were excluded from our study.

Cell lines and cell culture

The NSCLC cell line A549 was obtained from America Type Culture Collection and maintained in DMEM (Dulbecco's modified Eagle's medium), which was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C under a 5% CO₂ atmosphere.

Plasmid construction and cell transfection

The *SFRP1* expressing plasmid pEF6/V5-*SFRP1* and empty pEF6/V5 (negative control) were obtained from Yoshitaka Sekido (Nagoya University, Nagoya, Japan). Cells were transfected by these two types of vectors with FuGene HD Transfection Reagent (Roche, Mannheim, Germany) based on the instructions and maintained in normal medium.

RAN extraction and qRT-PCR analysis

RNA was first isolated from NSCLC tissues and adjacent normal tissues using Trizol reagent (Invitrogen) and then used to synthesize the first strand of cDNA through reverse transcription using a PrimeScript RT

reagent Kit (Takara, Dalian, China) following the manufacture's guides. RT-PCR reaction was performed with PRISM 7900 Sequence Detection System (Applied Biosystems). GAPDH served as the endogenous control. The relative mRNA expression of *SFRP1* was calculated by $-2^{\Delta\Delta CT}$ method. Each sample was in triplicate.

Immunohistochemistry (IHC)

IHC assay was performed to detect the expression of *SFRP1* at protein level. In brief, 4- μ m sections were prepared and then deparaffinized and rehydrated. Following, the sections were incubated with primary antibody and secondary antibody in order. Finally, all sections were stained with DAB and later counterstained by hematoxylin. Tissues were grouped according the percentages of stained cells. Those tissues with the percentage more than 30% belonged to the positive group and others were of the negative group.

Transwell assay

Transwell assay was used to investigate the role of *SFRP1* in migration and invasion of NSCLC cells. The transfected cells was first seeded into the upper chamber of the Transwell plates (BD Biosciences, San, Jose, CA, USA). Medium with 10% FBS was added into the lower chamber as a chemoattractant. After migration for 24h and invasion for for 48h, cells that migrated or invaded into the surface of the lower chamber were fixed by 90% alcohol, stained with 0.1% crystal violet and then counted in seven randomly selected fields. For invasion assay, the membrane should be per-coated by Matrigel (BD Biosciences, NJ, USA).

Statistical analysis

Statistical analyses were performed by SPSS 18.0 and Sigmaplot software. The differences between *SFRP1* expression and clinical factors were analyzed by students' t test. Chi-square test was adopted to estimate the relationship between *SFRP1* expression and clinical factors of NSCLC patients. The overall survival of NSCLC patients were compared with the Kaplan-Meier analysis. The prognostic significance of *SFRP1* was evaluated using the cox regression analysis. $P < 0.05$ was regarded to be statistically significant.

Results

The expression of *SFRP1* was decreased in NSCLC tissues at both mRNA and protein levels

The relative expression of *SFRP1* at mRNA and protein level were detected by qRT-PCR and IHC analyses, respectively. The results showed that the mRNA expression of *SFRP1* was significantly lower in NSCLC tissues than that in adjacent normal tissues (1.48 ± 0.38 vs 3.24 ± 0.51) (Fig. 1, $P < 0.05$). We further determined the expression of *SFRP1* at protein level in NSCLC tissues and normal controls using IHC method. The positive staining of *SFRP1* protein mainly presented in the cytoplasm of cells. As shown in

Table 1, the positive rate of *SFRP1* was 27.2% (91/125) in NSCLC tissues and 72.5% (37/51) in adjacent normal tissues, indicating that *SFRP1* expression at protein level was also down-regulated in NSCLC tissues ($P < 0.05$).

Table 1
The expression of *SFRP1* protein in NSCLC tissues and adjacent normal tissues

Samples	Case (n = 176)	SFRP1 expression		Positive rate	P value
		Positive	Negative		
NSCLC tissues	125	34	91	27.2%	< 0.05
Adjacent normal tissues	51	14	37	72.5%	

Relationship between *SFRP1* expression and clinical features of patients with NSCLC

The association of *SFRP1* expression and clinical features of NSCLC patients was listed and summarized in Table 2. It was found that the down-regulation of *SFRP1* was significantly associated with distant metastasis ($P = 0.043$), vascular invasion ($P = 0.021$) and TNM stage ($P = 0.013$). However, no correlation was observed between *SFRP1* expression and gender, smoking status and histology ($P > 0.05$).

Table 2
Relationship between *SFRP1* expression and clinical factors of patients with NSCLC patients

Clinical characteristics	Case (n = 125)	<i>SFRP1</i> expression		χ^2	<i>P</i> value
		Low	High		
Gender				0.388	0.533
Male	59	45	14		
Female	66	46	20		
Smoking status				1.829	0.176
Never	63	42	21		
Ever	62	49	13		
Histology				0.769	0.380
Adenocarcinoma	60	41	19		
Squamous cell carcinoma	65	50	15		
Vascular invasion				5.322	0.021
Present	67	55	12		
Absent	58	36	22		
Distant metastasis				4.078	0.043
Yes	68	44	24		
No	57	47	10		
TNM stage				6.182	0.013
I,II	65	54	11		
III,IV	60	37	23		

The effects of *SFRP1* on migration and invasion of NSCLC cells *in vitro*

A549 cells were transfected with pEF6/V5-*SFRP1* and pEF6/V5-NC. After transfection, the *SFRP1* expression was elevated by in cells with pEF6/V5-*SFRP1*. Then transwell assay was conducted to analyze the cell migration and invasion after being gtransfected. The outcomes demonstrated that the overexpression of *SFRP1* in viro significantly inhibited the migration and invasion of NSCLC cells compared to that in cells transfected with pEF6/V5-NC (Fig. 2A and Fig. 2B, $P < 0.05$).

The prognostic value of *SFRP1* in patients with NSCLC

To further examine the correlation between *SFRP1* expression and overall survival of NSCLC patients, a 5-years' follow-up was conducted. Based on the data of follow-up, Kaplan-Meier analysis was carried out. It displayed that patients with low *SFRP1* expression had a significantly shorter overall survival than those with high expression (Fig. 3, log rank test, $P < 0.001$). Besides, univariate and multivariate analyses adjusted for clinical factors using cox regression analysis was performed to assess the prognostic value of *SFRP1* in NSCLC. As shown in Table 3, vascular invasion ($P = 0.002$, HR = 2.170, 95%CI = 1.344–3.504), TNM stage ($P = 0.008$, HR = 1.892, 95%CI = 1.184–3.023) and *SFRP1* expression ($P = 0.000$, HR = 4.860, 95%CI = 2.407–9.813) were identified as important prognostic markers in NSCLC (Table 3).

Table 3
Univariate and multivariate analyses adjusted for clinical factors for estimating the prognostic value of *SFRP1* in NSCLC

Clinical factors	Univariate		Multivariate	
	<i>P</i> value	HR(95%CI)	<i>P</i> value	HR(95%CI)
Gender (male vs female)	0.527	0.862(0.545–1.364)	-	-
Smoking status (Never vs Ever)	0.040	1.621(1.023–2.569)	-	-
Histology (Adenocarcinoma vs Squamous cell carcinoma)	0.726	1.085(0.687–1.713)	-	-
Vascular invasion (Present vs Absent)	0.003	2.129(1.293–3.507)	0.002	2.170(1.344–3.504)
Distant metastasis (Yes vs No)	0.116	1.442(0.913–2.276)	-	-
TNM stage (I,II vs III,IV)	0.013	1.864(1.142–3.044)	0.008	1.892(1.184–3.023)
<i>SFRP1</i> expression (Low vs High)	0.000	3.779(1.841–7.754)	0.000	4.860(2.407–9.813)

Discussion

NSCLC is the most lethal human cancer and almost half of patients with newly diagnosed NSCLC have metastatic [21]. Despite great progress have obtained in the diagnosis and treatments, the prognosis of NSCLC remains poor as it often at advanced stages when diagnosed [22, 23]. It is therefore of great

importance to find some effective and accuracy bio-markers for the diagnosis and prognosis of NSCLC so that provide new therapy target for this cancer.

Recently, attention is shifted onto candidate molecules for radical treatment of various cancers. So far, a variety of biomarkers have been discovered to function as indicators in NSCLC. According to Zhan et al., high expression of *Orai1* was proved to be a promoter for cell proliferation and predicted a poor prognosis in NSCLC [24]. Fan et al., reported that *ELMO3* was a candidate prognostic and diagnostic predictor for NSCLC [25]. Luo et al., revealed that FSCN1 played crucial roles on the development of NSCLC and might act as an efficient biomarker for patients with this disease [26].

SFRP1 was expressed in various tissues, such as the brain, kidney and heart, and its transcript was present both in the adult and during embryogenesis [27, 28]. It was also reported to abnormal expression in various of diseases. For instance, Rogler et al., found the expression of *SFRP1* was inhibited by hypermethylation and it could predict the outcome of patients with bladder cancer [29]. Zheng et al., claimed the expression of *SFRP1* was decreased and it could be a prognostic marker combining with the expression of β -catenin in prostate cancer [30]. The methylation of *SFRP1* was proved to be an useful diagnostic indicator in cholangiocarcinoma [31]. An et al., detected the expression of *SFRP1* and its down-regulation was related to the progression of acute myeloid leukemia [32]. In the present study, we measured the *SFRP1* expression and found it was decreased in NSCLC tissues compared to that in adjacent normal controls. The result suggested *SFRP1* was a tumor suppressor in NSCLC. All our findings were in accordance with the previous reports.

Then we analyzed the relationship between *SFRP1* expression and clinical characteristics of NSCLC patients. The low *SFRP1* expression appeared to be significantly associated with vascular invasion, distant metastasis and TNM stage, suggesting that *SFRP1* expression might be involved in the development and progression of NSCLC. Based on this, we further investigated the role of *SFRP1* on cells migration and invasion by transwell assay. The up-regulation of *SFRP1* was considered to significantly inhibited migration and invasion of NSCLC cells.

Subsequently, we estimated the prognostic value with the data from follow-up. Kaplan-Meier analysis displayed that patients with high *SFRP1* expression had a longer overall survival than those with low expression. What's more, cox regression analysis demonstrated that *SFRP1* was a novel biomarker for the prognosis of NSCLC patients. However, although the prognostic role in NSCLC has been validated, the precise mechanism of *SFRP1* on development and progresses of NSCLC is still unclear and a sophisticated issue. It is known to all that Wnt signaling pathway not only affects the expression of genes but also influences cell migration [33, 34]. As a newly discovered antagonist of Wnt signaling pathway in recent years, *SFRP1* has important inhibitory effects on the transmission of Wnt pathway [35]. Therefore, we inferred that *SFRP1* might function on NSCLC through the Wnt signaling pathway, which still need more and further studies.

Conclusions

All in all, the expression of *SFRP1* is decreased and it participates in the development and progression of NSCLC via regulating the cell migration and invasion. Moreover, the low expression of *SFRP1* is confirmed to be an independent prognostic bio-marker in NSCLC. However, its mechanism is unclear and the sample scale is small in this study. Some further more studies are need in future.

List Of Abbreviations

secreted fizzled-related protein-1 (*SFRP1*)

non-small cell lung cancer (NSCLC)

quantitative real-time polymerase chain reaction (qRT-PCR)

tumor node metastasis (TNM)

fetal bovine serum (FBS)

Immunohistochemistry (IHC)

Declarations

Ethics approval and consent to participate

This study was supported by the Ethics Committee of Cangzhou Central Hospital and also has been carried out in accordance with the World Medical Association Declaration of Helsinki.

The subjects had been informed the objective. Certainly, written consents were signed by every subject in this study.

Consent for publication

We obtaining permission from participants to publish their data.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Authors' contributions

Y.Z. design of the work; X.K. the acquisition, analysis, Y.Z. interpretation of data; X.K. the creation of new software used in the work; H.W. have drafted the work or substantively revised it. All authors read and approved the final manuscript.

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Figures

Figure 1

The relative expression of SFRP1 at mRNA level in NSCLC tissues and adjacent normal controls was determined by qRT-PCR. SFRP1 mRNA expression was down-regulated in NSCLC tissues compared to that in adjacent normal controls ($P < 0.05$).

Figure 2

The effects of SFRP1 on cell migration and invasion of NSCLC cells in vitro. A: The up-regulation of SFRP1 suppressed the cell migration ($P<0.05$). B: The increased of of SRFP1 inhibited the cell invasion ($P<0.05$).

Figure 3

Correlation of SFRP1 expression and overall survival of NSCLC patients. Patients with low SFRP1 expression presented a notable poorer prognosis compared to those with high expression, hinting SFRP1 expression was a candidate predictor for NSCLC patients (log rank test, $P < 0.001$).