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Transcriptional inactivation of secreted frizzled-related protein 1 by promoter hypermethylation as a potential biomarker for non-small cell lung cancer

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Epigenetic silencing of secreted frizzled-related protein (SFRP) genes, antagonists of the WNT pathway, contributes to the pathogenesis of several cancers including non-small cell lung cancer (NSCLC). We hypothesize that methylation analysis of SFRPs family could improve their use as a panel of biomarkers for diagnosing and staging of NSCLC in China. The expression of four SFRP members (SFRP1, 2, 4, and 5) in NSCLC samples was screened by RT-PCR and quantitative real-time PCR. Only SFRP1 was significantly downregulated in NSCLC, as compared to adjacent normal tissues and benign pulmonary disease tissues (P=0.006). Promoter hypermethylation of SFRP1 was found in 32.1% (25/78) NSCLC specimens and was closely correlated with loss of expression, besides SFRP1 hypermethylation was associated with lymph metastasis (P=0.039) and disease progression within one year (P=0.027). Furthermore, methylated SFRP1 was detected in 28.2% (22/78) of plasma samples from NSCLC patients while only 4% (2/50) in cancer-free controls, and the concordance of SFRP1 methylation status in tumor tissues and corresponding plasmas was satisfactory (P<0.001). In conclusion, epigenetic inactivation of SFRP1 is a common event contributing to lung carcinogenesis and maybe used as a potential biomarker for NSCLC in Chinese population.

Key words: SFRP 1; DNA methylation; biomarker; non-small cell lung cancer

Lung cancer is the leading cause of cancer-related deaths in the world [1]. In China, the incidence and mortality rates have been increasing rapidly in the last two decades. Despite new diagnostic techniques, most lung cancers are detected late, with an overall 5-year survival rates remaining below 18% and 7% in non–small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), respectively. The poor prognosis of lung cancer patients is largely a result of the occult metastatic dissemination, which appears in approximately two-thirds of patients at the time of diagnosis. When early stage lung cancer is detected, the survival rate can increase dramatically [2]. Thus, the development of efficient diagnostic methods to enable the early detection is clearly imperative. Molecular markers would provide an alternative approach and among them, DNA methylation alterations in the promoter region of tumor suppressor genes (TSGs) show great promise [3]. Moreover, it has been demonstrated that methylated DNA can be isolated from "remote media", such as blood, sputum, or bronchoalveolar lavage (BAL), making it well suited for non-invasive detection [4, 5].

Secreted frizzled-related proteins (SFRPs), a family of five secreted glycoproteins, are extracellular signaling molecules that antagonize the Wnt/β-catenin pathway, which is known to be involved in embryogenesis and tumorigenesis [6]. Downregulation and methylation of SFRPs gene have been identified in a variety of malignancies, including gastric cancer, hepatocellular cancer, esophageal cancer, breast cancer, and leukemia [7–11], which strongly suggests SFRPs function as tumor suppressor genes. As for lung cancer, aberrant methylation of SFRP1 (34%), SFRP2 (52%), and SFRP5 (33%) was found in 238 malignant lung tissues [12]. In another study, epigenetic alteration of the SFRPs promoter region in 146 NSCLC was analysed and hypermethylation of 76% for SFRP1, 84% for SFRP2, 29% for SFRP4, and 69% for SFRP5 was detected [13]. These findings give us interests to evaluate the SFRPs hypermethylation as a promising molecular marker for diagnosis and staging of Chinese lung cancer patients.

Because NSCLC is the less aggressive lung cancer subtype, and accounts for 85-90% of all cases, we focus on NSCLC patients in the present study. The expression level and methyla-

tion status of SFRPs were determined in 78 pairs of NSCLC and adjacent non-cancer tissues, and the potential use of detecting methylated SFRP DNA in plasma as a biomarker for NSCLC was further evaluated.

Materials and methods

Study population. A total of 78 paired primary NSCLC specimens and their adjacent normal tissues, 25 no-cancer lung tissues from patients with benign pulmonary diseases were obtained in Departments of Cardiothoracic Surgery, Jinling Hospital from Nov 2007 to June 2008. All specimens were immediately snapped frozen in liquid nitrogen and stored at –80°C until use. Meanwhile, plasma samples were collected from the same cancer patients and 50 controls of benign pulmonary diseases or healthy donors. The age of the patients ranged from 35 to 80, with a median 59 and the average 60.3, and the numbers of them in stage I , II, III, IV were 25, 33, 19, 1 (brain metastases), respectively. None received preoperative chemotherapy or radiation therapy. All diagnoses were based on pathological and/or cytological evidence. Histological classification was conducted according to the 1999 "Histological typing of lung and pleural tumors: third edition" of the WHO, and tumor stage was determined according to the 2003 TNM staging guideline suggested by the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC). Ethical approval was obtained from the hospital and fully informed consent from all patients prior to sample collection.

Reverse transcription, polymerase chain reaction (RT–PCR) and Quantitative real-time PCR. Since SFRP3 does not contain CpG-islands in its promoter region [14], we only examined the gene expression levels of SFRP1, SFRP2, SFRP4, and SFRP5 in this study. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription reaction was performed using 2 μg of total RNA with a first strand cDNA kit (Takara, Shiga, Japan), according to the manufacturer's instructions. Polymerase chain reaction was run in a 25 μl volume containing 2 μl of cDNA template, 10×Buffer, 0.15 mM dNTP, 0.1 mM each primer and 0.5U of Ex Taq Hot Start Version (Takara). All the primer sequences and PCR amplification conditions are described elsewhere [15]. Quantitative real-time PCR was performed in ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA), using the SYBR Premix Ex Taq™ kit (Takara). Data analysis was done using the $2^{-\Delta\Delta CT}$ method for relative quantification [16], and all samples were normalized to GAPDH, which was used as an endogenous control.

Bisulphite treatment of DNA, methylation-specific polymerase chain reaction (MSP). Genomic DNA from tissues and plasma was extracted using QIAamp DNA Mini Kit and QIAamp Blood Kit (Qiagen, Hilden, Germany), respectively. Extracted DNA was quantified spectrophotometrically, and around 300 ng DNA was extracted from 2ml cancer patients plasma. Methylation statuses of SFRP1 were determined by MSP. Briefly, 0.2~1 μg of genomic DNA was bisulphite-treated with EZ-DNA methylation Gold Kit (Zymo Research, Orange, CA, USA). Then 2 μl of bisulphite-treated DNA was used as a template for MSP. Primers of methylated and unmethylated SFRP1 seen in elsewhere [9], PCR mixture system performed refer to above. Lymphocyte DNA, original or methylated in vitro by excessive CpG (SssI) methylase (New England Biolabs, Beverly, MA, USA) following the manufacturer's directions, was used as unmethylation and methylation positive control. Water blank was used as a negative control. To verify the MSP results, stochastic bands from each target were gel-purified and cloned into pMD 18-T Vector (Takara) followed by automatic DNA sequencing provided by GeneScript (Nanjing, China).

Follow-up. After tumor excision, 54 patients treated with four to six times of first-line platinum-based combination chemotherapy as an adjuvant therapy were included. The diease progresssion (recurrence, regional lymph metastasisas, distant metastis, or death) or not within one year was evaluated by imaging evidence.

Statistical analysis. The results were expressed as mean±s.d. or percentage where appropriate. The differential expressions of SFRPs mRNA between cancer and non-cancer specimens were calculated with Mann–Whitney *U*-test. Differences in frequency were assessed by Chi-square test or Fisher's exact test. Statistical tests were carried out using SPSS version 12.0 for windows (SPSS Inc., Chicago, IL, USA). $P<0.05$ was taken as statistical significance.

Results

Expression of SFRPs mRNA in NSCLC and adjacent normal lung tissues. We first determined the mRNA expression of SFRPs in 78 pairs of NSCLC tissues by RT-PCR (Figure 1A) and quantitative real-time PCR (Figure 1B). The results showed that, in 41 cases (52.6%) was detected a marked downregulation of SFRP1 in NSCLC specimens, compared to adjacent normal tissues and no-cancer lung tissues from patients with benign pulmonary diseases ($P=0.006$). However, the downregulation was not observed in SFRP2, SFRP4, and SFRP5. Furthermore, SFRP4 was significantly upregulated in 71.8% (56/78) of NSCLC specimens ($P<0.001$), while SFRP5 was mostly silenced (60.3%, 47/78) both in tumor and adjacent normal lung tissues.

Methylation status of SFRP1 in NSCLC and adjacent normal lung tissues. To investigate the role of promoter methylation in silencing of SFRP1 in NSCLC, the methylation status of SFRP1 was analyzed by MSP. We found that 32.1% (25/78) of NSCLC specimens had SFRP1 methylation, which was significantly higher than that of adjacent normal tissues (7.7%, 6/78) ($P<0.001$) (Table 1) (Fig 2). Notably, aberrant methylation of SFRP1 was not detected in lung tissues from 25 non-cancer controls. What's more, among the patients with SFRP1 downregulation, 53.7% (22/41) showed a promoter methylation, and within all of the methylation cases, 88% (22/25) showed SFRP1 downregulation, whereas in unmethylated cases, only

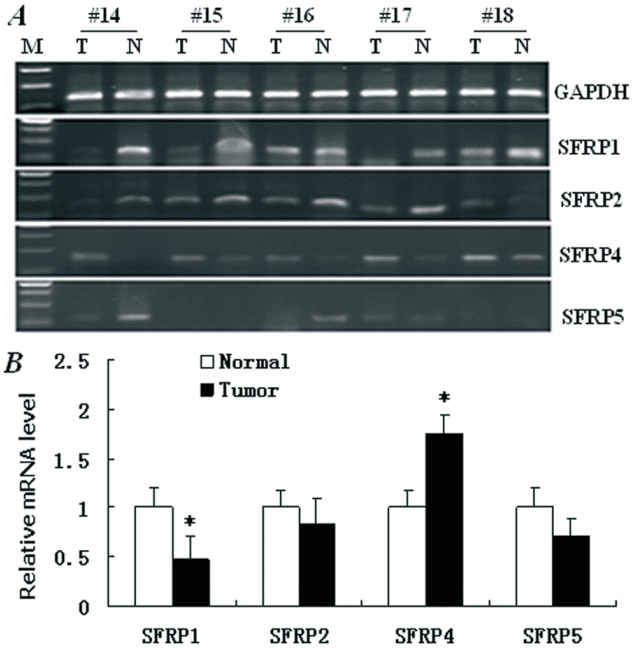

Figure 1. Transcript expression of SFRPs in NSCLC tumor and adjacent normal lung tissues. (A) typical RT-PCR results of mRNA expression levels of SFRPs in five matched pairs (patients14-18) of primary NSCLC (T) and their adjacent normal lung tissues (N). GAPDH was used as an endogenous control. Only SFRP1 gene was detected a low or absent of transcripts in most tumors, compared to adjacent normal tissues and non-cancer lung tissues from controls. (B) mRNA expression levels of SFRPs in NSCLC (Tumour) and their adjacent normal tissues (Normal) as determined by quantitative real-time PCR (n=78). The results were expressed as the ratio of copies of target gene relevant to GAPDH form three independent experiments. Data are expressed as mean±s. d., *P<0.05.

Table 1 SFRP1 downregulation in NSCLC tissues was associated with promoter methylation

NSCLC tissues	SFRP1 expression		P-value
	down	-/up	
Methylation	22	3	0.00002
Unmethylation	19	34	

35.8% (19/53) showed suppression of SFRP1. SFRP1 methylation was tightly correlated with loss of expression. (P<0.001) (Table 1).

Clinicopathological correlation of SFRP1 hypermethylation in NSCLC specimens. The relationship between SFRP1 promoter hypermethylation status and clinicopathological features was analyzed. As showed in Table 2, SFRP1 methylation in NSCLC was associated with lymph metastasis and disease progression, in patients with lymph metastasis, the frequency of SFRP1 methylation (44.1%, 15/34) was higher than that without lymph metastasis(22.7%, 10/44)(P=0.039). Further more, in 54 patients with reliable follow-up data, 33 cases had progressed within one year after surgery (recurrence, 10; regional lymph metastasisas and/or distant metastis, 20; death,3) and 21 cases were of stable disease. The methylation status of these two group was significantly different (16/33 vs 4/21, P=0.027). However, there were no correlation with patient gender, age, histological type, cellular differentiation and smoking habit. Although methylation of SFRP1 was more frequent in III/IV stage (45%, 9/20) than that in I/II stage(27.6%, 16/58), no statistical significance was found, which maybe due to the small samples of III/IV stage.

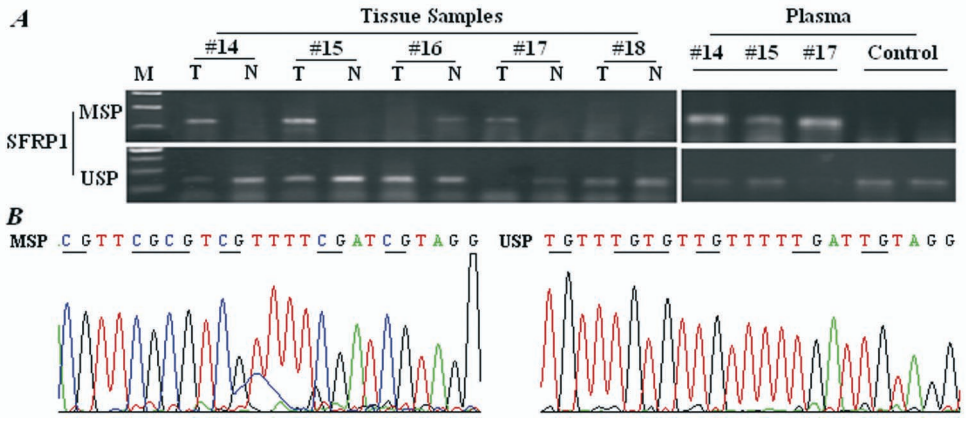

Figure 2. Methylation status of SFRP1 gene in matched tumor tissues and plasma samples of NSCLC patients. (A) typical agarose gel electrophoresis of MSP results. T, NSCLC tumor; N, adjacent normal lung tissues (patients14-18). Three plasma DNA samples came from NSCLC patients (patients14, 15 and 17) and two from controls of benign pulmonary diseases. SFRP1 showed a hypermethylation in tumor in the patients with SFRP1 downrugulation. SFRP1 methylation status in plasma samples was in accordance with corresponding tumor tissues. (B) The MSP product of SFRP1 was directly sequenced and confirmed. Methylated cytosines (C) would not be converted to uracil (T) and remained as C.

Table 2 SFRP1 methylation in NSCLC plasmas was in concordance with corresponding tumor tissues

NSCLC tissues	SFRP1 methylation in plasma		*P*-value
	Methylation	Unmethylation	
Methylation	21	4	0.00000
Unmethylation	1	52	

Methylation status of SFRP1 in plasma samples of NSCLC patients. To further investigate whether SFRP1 methylation could be used as a biomarker for NSCLC, corresponding plasma samples from NSCLC patients were tested for methylated status of SFRP1. A total of 22 (28.2%) out of 78 NSCLC plasmas showed SFRP1 methylation whereas only 2 (4%) in the 50 cancer-free controls ($P<0.001$). Moreover, 21 cases showed methylated alteration both in tumor tissues and plasma samples, with a sensitivity of 84% (21/25) and a specificity of 98.1% (52/53) (Table 2) (Fig 2), the concordance of SFRP1 methylation status in tumor tissues and corresponding plasma samples was well ($P<0.001$).

Discussion

The inappropriate activation of Wnt/β-catenin signalling contributes to the pathogenesis of several human malignancies. The function of SFRP family as antagonist of the Wnt pathway provides a potential mechanism to suppress the abnormal activation of this pathway. Previous studies have shown SFRPs downregulation in many cancers. However, in different human tumors, the expression pattern of SFRPs is not exactly the same. There may be functional differences among SFRP family genes [17]. As our results indicated, SFRP1 was significantly downregulated in NSCLC specimens, while SFRP2, SFRP4, and SFRP5 was not. It did not fully accord with other reports [12,13], the contradiction may be due to sample heterogeneity. In particular, we found an overexpression of SFRP4 in NSCLC first time. SFRP4 overexpression was also found in primary prostate carcinomas [18], endometrial stromal sarcomas [19], and colorectal carcinomas [20]. The molecular mechanisms responsible for the overexpression of SFRP4 and the effect on tumors are not well studied. However, increased levels of SFRP4 in tumor samples are evidence against the hypothesis that SFRP4 functions as a tumor suppressor in these tumor models.

Since only SFRP1 was downregulated, we extended the study to determine the methylation status of SFRP1 in NSCLC specimens, because aberrant promoter hypermethylation was one of the primary mechanisms in the down-regulation of tumor suppressor genes, and SFRP1 promoter methylation had been detected in a variety of human solid tumors [21–23]. We found 25 out of 78 NSCLC specimens had SFRP1 methylation. The frequency was relatively lower than the studies of Marsit et al [13] and Fukui et al [24], but similar to Suzuki et al [12]. Our data also indicated that, promoter methylation of SFRP1 was specifically associated with low or absent mRNA transcripts in NSCLC. Furthermore, SFRP1 expression was reported to be restored in NSCLC cell lines by the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-CdR) treatment, and ectopic expression of SFRP1 caused the reduction of cells colony formation and inhibited the transcriptional activity of β-catenin [24]. Together with many other results [25, 26], we could conclude that, SFRP1 acts as a functional tumour suppressor gene in NSCLC and transcriptional inactivation of SFRP1 by promoter methylation is a common event contributing to lung carcinogenesis in Chinese population.

Recent epigenetic studies suggest that silencing of the TSGs by DNA hypermethylation at CpG islands tend to be an early event in the multi-step pathway of carcinogenesis. A steady increase in the frequency of Wnt antagonists methylation has

Table 3 Association between the SFRP1 hypermethylation in NSCLC specimens and clinicopathological features

Patients	Cases	SFRP1 methylation		*P*-value
		Methylated	Unmethylated	
Sex				0.433
Male	58	20	38	
Female	20	5	15	
Age				0.203
<60	22	5	17	
≥60	56	20	36	
Histological type				0.739
Adenocarcinoma	30	11	19	
Squamous cell carcinoma	36	10	26	
Others	12	4	8	
Cellular differentiation				0.265
Well	13	4	9	
Moderately	46	14	32	
Poor/undifferentiated	19	7	12	
Stage				0.124
I/II	58	16	42	
III/IV	20	9	11	
Lymph metastasis				0.039*
Negative	44	10	34	
Positive	34	15	19	
Smoking				0.348
Yes	49	17	32	
No	29	8	21	
Progession within one year				0.043*
Yes	33	16	17	
No	21	4	17	

Chi-square test or Fisher's exact test, *$P<0.05$

been found in normal parenchyma, glandular neoplasia, and adenocarcinomas of lung [27]. It thus appears that epigenetic alteration of SFRPs is present in the early stage of lung cancer. Based on the frequency of tumor-specific hypermethylation of SFRP1 in NSCLC specimens, we suspected that it could be used as a valuable marker for early diagnosis. The detection of SFRP1 methylated status in plasma samples of NSCLC patients was further implemented, whilst we have previously known that methylated DNA could be detected in cancer patients' peripheral blood [28]. We found SFRP1 was hypermethylated in 22 plasma samples out of 78 NSCLC patients, whereas only 2 detected in 50 cancer-free controls, suggesting plasma SFRP1 methylation scan is a promising diagnostic technique for NSCLC. The sensitivity was satisfactory compared with several other frequently methylated loci identified in plasma/serum, for example CDKN2A/p16, CDH13, DAPK, MGMT, RARβ and RASSF1A [4, 29–32]. It is of note that concordance of SFRP1 methylation status in tumor tissues and corresponding plasma samples is fine, which further implies its potential diagnostic value in NSCLC.

Our results also showed SFRP1 hypermethylation in NSCLC specimens was associated with lymph metastasis and disease progression within one year after surgery, which hinted us that SFRP1 methylation maybe used as a staging or prognosis factor [33]. We presume that epigenetic aberrant methylation of SFRP1 is not only an important mechanism leading to malignant transformation, but also persists and aggravates with the progression of NSCLC. On the other hand, it may participate in the alteration of sensitivity to chemotherapeutic drugs as well, because patients with poor treatment effectiveness was higher in methylation of SFRP1. Epigenetic modification occuring during the acquisition of drug resistance has been confirmed by mamy repotrs [34], though the molecular mechanism of which needs further study, so SFRP1 methylation may be a useful tool to assay therapy and predict the efficacy of promising chemopreventive agents for NSCLC. There is no doubt that the personalized cancer therapy brings good news to patients, when SFRP1 methylation is detected, clinicians should be alert about the possibility of advanced conditions of tumor and adopt better management for patients, if the preliminary findings in the present study could be further substantiated in long-term and large sample surveys.

However, researches of methylation markers for lung cancer diagnosis, prognosis, and chemotherapeutic prediction are ongoing now, the analyses of DNA methylation in remote media are still in their early stages. Many loci examined show low sensitivity, even for the more frequently methylated loci identified by new approaches [35, 36], DNA methylation at a single gene cannot be expected to detect all cases of the cancer. The way to address this problem is to identify a panel of multiple loci with more sensitivity and specificity. Take together, our study confirms epigenetic inactivation of SFRP1 is a common event contributing to lung carcinogenesis, and provides a potential biomarker constituting the methylation-based panal for NSCLC in Chinese population.

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