Int J Clin Exp Pathol 2017;10(5):6064-6070
www.ijcep.com /ISSN:1936-2625/IJCEP0030528

Original Article

Aberrant loss of SFRP1 expression is associated with poor prognosis in pancreatic cancer

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Received April 14, 2016; Accepted June 15, 2016; Epub May 1, 2017; Published May 15, 2017

Abstract: The secreted frizzled-related protein 1 (SFRP1), a 35 kDa secreted glycoprotein, has been considered a modulator of Wnt signaling. Accumulating evidence show that aberrantly decreased or loss of SFRP1 is associated with increased proliferation, metastasis, as well as, poor clinical prognosis in various human cancers. However, the role of SFRP1 in the pancreatic cancer is not clarified yet. In this study, we investigated SFRP1 expression in pancreatic cancer cases through quantitative real-time PCR and tissue microarray analysis to evaluate the clinical significance of SFRP1 expression. Pancreatic cancer prognosis was analyzed by Univariate and Kaplan-Meier analysis. Consequently, compared with paired adjacent non-tumorous tissues, SFRP1 mRNA or protein expression was down-regulated in primary tumor tissues. Decreased SFRP1 expression was significantly associated with pT classification and exhibited poor prognosis in pancreatic cancer. Cox proportional hazards regression modeling analysis revealed that lymph node metastasis (P = 0.027; hazard ratio, 1.850, 95% CI, 1.074-3.185), tumor differentiation (P = 0.030; hazard ratio, 0.547, 95% CI, 0.317-0.945) and SFRP1 expression (P = 0.035; hazard ratio, 0.441, 95% CI, 0.206-0.942) were three independent prognostic factors. Our findings suggest that SFRP1 is associated with pancreatic cancer susceptibility and prognosis and SFRP1 may potentially serve as biomarkers for the prediction of prognosis in pancreatic cancer.

Keywords: SFRP1 protein, pancreatic neoplasms, clinical pathology, prognosis

Introduction

Pancreatic cancer is one of the malignant tumor with aggressive growth, characterized early invasion and metastasis [1, 2]. Due to its obvious lack of effective chemotherapeutic options and only 15-20% resection rate [3, 4], pancreatic cancer is dismal disease with a overall 5-year survival of 5% [5, 6]. Hence, there is a great need additional information for diagnosis and novel therapeutic approaches for improving prognosis. Recent a remarkable finding in the process of pancreatic cancer is that aberrant activation of Wnt pathway occurs in almost all pancreatic cancer.

The secreted frizzled-related protein 1 (SFRP1), a 35 kDa secreted glycoprotein, is localized to chromosome 8p12-p11.1. SFRP1 belongs to secreted frizzled-related proteins (SFRPs) family, which contain a cysteine-rich domain (CRD) with similarity to the frizzled (Fz) membrane reception, a Wnt ligands binding domain [7]. To compete with Fzs for Wnt ligands or bind directly to Wnt [8], SFRPs indirectly degrade and reduce levels of the transcription factor beta-catenin, which regulates of downstream target genes, to antagonize the Wnt signaling pathway involved in the development of cancer. Previous studies have shown that the expression of SFRP1 was decreased in several types of digestive system cancer [9-11]. To date, SFRP1 is down-regulated by promoter hypermethylation in various human cancers [12, 13]. Increasing evidences have indicated that SFRP1 could inhibit the growth of different types of cancer cells in vivo and vitro, including cervical [14], lung [15], and liver [16]. Recent studies have shown that SFRP1 gene is common hypermethylation and aberrant expression in pancreatic carcinogenesis [17]. SFRP1 mRNA loss may be involved in the process of pancreatic carcinogenesis [18].

In the current study, we detected the expression of SFRP1 messenger RNA (mRNA) and pro-

tein in clinical primary pancreatic cancer materials and analyzed the correlations with clinicopathological features and disease outcomes. This study aimed to evaluate the importance of SFRP1 expression in pancreatic carcinogenesis and suggest a potential role of SFRP1 as a prognostic marker for pancreatic cancer.

Materials and methods

Patients and specimens

A total of 90 patients who had resectable duct adenocarcinoma of the pancreas were enrolled in this research project. All patients underwent surgical pancreatic resection at the PLA General Hospital in Beijing, China, from 2005 to 2008 and had not received preoperative radiation therapy or chemotherapy. The ratio of males to females was 59:31 and the mean age was 62 years. Primary pancreatic cancer tissues and paired adjacent non-tumor tissues were collected from these patients who gave informed consent to use excessive pathological specimens for research purposes. The tumor pathological grade was assessed according to WHO criteria, and staged following UICC/AJCC tumor-node-metastasis (TNM) classification of malignant tumors [19]. The patient follow-up data was completed on November 2013 and the follow-up period range 1-60 months. This study was approved by the Chinese PLA General Hospital's Ethics Committee.

Quantitative transcription-polymerase chain reaction (qRT-PCR) analysis

Quantitative transcription-polymerase chain reaction was used to evaluate the mRNA expression levels of SFRP1. In brief, total RNA of tissues was extracted using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol, and then reverse transcribed to first-strand complementary DNA (cDNA) using the Reverse Transcription System Kit (Promega, Madison, WI). qRT-PCR was performed with 7500 Real-Time PCR System (Applied Biosystems, CA, USA). Relative levels of mRNA expression were used as normalization controls for SFRP1 mRNA and calculated according to the $2^{-\Delta\Delta CT}$ method as previously described [20]. Primer sequences for the genes analyzed as follows: SFRP1 forward, 5'-ACGTGGGCTACAAGAAGATGG-3'; SFRP1 reverse, 5'-CAGCGACACGGGTAGATGG-3'; GADPH forward, 5'-CTTTGGTATCGTGGAAGGACTC-3'; GADPH reverse, 5'-GTAGAGGCAGGGATGATGTTCT-3'.

Tissue microarray construction and immunohistochemical (IHC) staining

Tissue microarrays were constructed from the formalin-fixed paraffin-embedded tissue blocks of 180 tissues samples, including 90 tumor and 90 paired adjacent non-tumor tissues specimens. The cores (1.5 mm size) were punched from the selected tissues and harvested into recipient blocks using microarray punching instrument.

The serial number of 5 μm thick tissue sections cut from the tissue blocks were mounted on the slide. In bref, the slide baked at 65°C for 90 min. After deparaffinization and rehydration, the sections were incubated with 3% hydrogen peroxide for 30 min. For antigen retrieval, the slides were boiled in the steam boiler with preheating sodium citrate buffer (10 mM, pH 6.0) for 15 min. The slides treated with 10% normal goat serum at 37°C for 30 min to block nonspecific binding and then incubated with rabbit polyclonal antibody against SFRP1 (Abcam Inc.) at dilution of 1:150 at 4°C overnight. For negative control, replacement of primary antibody by phosphate buffer solution (PBS) was used as blank control. The slides were then incubated with secondary antibodies that were goat anti-rabbit immunoglobulins (Santa Cruz Biotechnology Inc) and then stained with diaminobenzidine tetrahydrochloride (DAB). The nuclei were counterstained with hematoxylin.

Immunohistochemistry analysis

The staining intensity and extent of the staining area were graded according to the Amend Allred scoring system as described previously [20]. Staining intensity was scored as follows: (no staining = 0; weak staining = 1; moderate staining = 2; strong staining = 3); The percentage of positive tumor cells was sored as follows: (< 5% = 0, 5-24% = 1, 25-49% = 2, 50-74% = 3, 75-100% = 4). The expression of SFRP1 determined by multiplying intensity score to the percentage of stained cells score. Tissues with immunohistochemical scoring ≤ 4 were considered as low expression and with scoring > 4 as high expression.

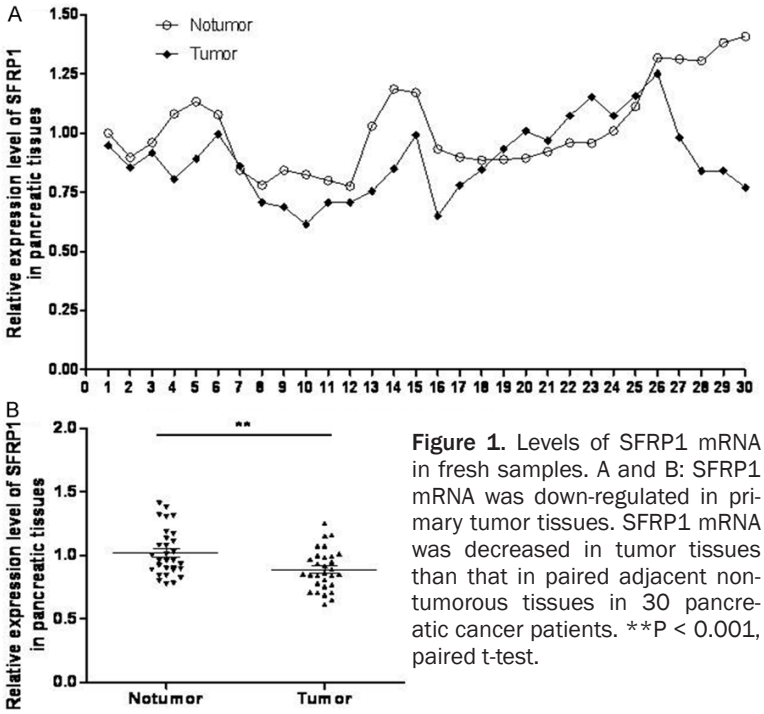

Figure 1. Levels of SFRP1 mRNA in fresh samples. A and B: SFRP1 mRNA was down-regulated in primary tumor tissues. SFRP1 mRNA was decreased in tumor tissues than that in paired adjacent non-tumorous tissues in 30 pancreatic cancer patients. **P < 0.001, paired t-test.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Windows software (SPSS, Chicago, IL, USA). All date are presented as means ± SD. Student's t-test was used to compare two means and paired t-test were used to evaluate the expression of SFRP1 mRNA in primary tumorous and surgical margin tissues. The chi-square test or Fisher's exact test were employed to analyse the association of clinicopathologic parameters and SFRP1 expression. Survival curves were calculated by Kaplan-Meier method, and analysis was using the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. *P*-Values < 0.05 were considered statistically significant.

Results

SFRP1 is frequently reduced in pancreatic cancer

SFRP1 was found frequently methylated and reduced in several cancer tissue [10, 21], Including pancreatic cancer cell line [17]. To determine whether the expression level of SFRP1 reduced in pancreatic cancer tissue. We first examined SFRP1 mRNA expression in 30 pairs of tissues, primary tumorous and surgical margin tissues, by using qRT-PCR. Results showed that SFRP1 mRNA was frequently lower than most non-tumor counterparts (**Figure 1A**). And the relative mRNA expression level of SFRP1 was obviously down-regulated in primary tumor, compared with surgical margin non-tumor counterparts (P = 0.001, t-test; **Figure 1B**). To further validate the result of SFRP1 exression, IHC was then performed to examine SFRP1 expression in 90 cases of primary tumors and paired surgical-margin non-tumor tissues (**Figure 2**). Amend Allred scoring system showed that high expression of SFRP1 was found in 82 (82/90, 91.1%) pancreatic surgical margin non-tumor tissues and in 26 (26/90, 28.9%) tumor tissues. SFRP1 expression was significantly lower in tumor samples than that in surgical margin non-tumor samples (P < 0.001, χ^2 test; **Figure 2**).

Decreased SFRP1 expression predicted a poor prognosis in pancreatic cancer patients

On the basis of the SFRP1 expression in tumor tissues, 90 patients with pancreatic cancer were divided into tow group: SFRP1 high-expression (n = 26) and low-expression group (n = 64). The correlations between SFRP1 expression and clinicopathologic features were further summarized and analyzed (**Table 1**). SFRP1 expression inversely associated with pT classification (P = 0.036). However, there was no significant correlation between SFRP1 expression with patient's age, gender, lymph node metastasis status, perineural invasion, tumor differentiation, smoking status (**Table 1**).

Survival data were available from all patients with pancreatic cancer. In present study, we evaluated the associations between SFRP1 expression and overall survival (OS) in 90 patients. Long-rank test showed that low SFRP1 expression in patients with pancreatic cancer was associated with a worse prognosis (P <

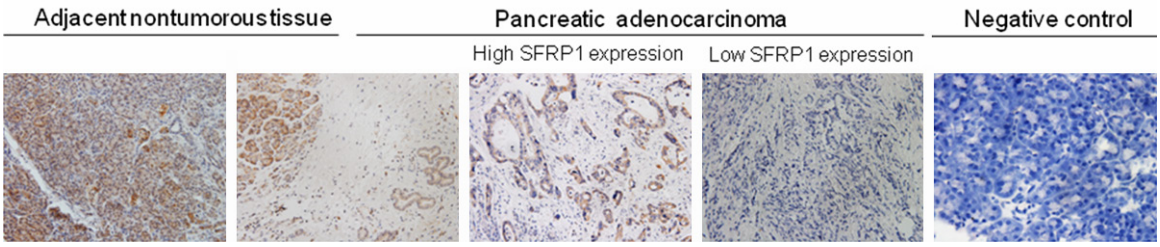

Figure 2. Representative images of immunohistochemical staining of SFRP1 in adjacent non-tumorous tissue and pancreatic tumor tissues. The left image showed SFRP1 staining in normal cells (original magnification × 200). The left second image showed SFRP1 staining in section with adjacent non-tumorous tissue and tumor tissues (original magnification × 200). The right middle tow images showed different level of SFRP1 in pancreatic cancer cells (original magnification × 200). The right image was the negative control (original magnification × 400).

Table 1. Correlation between SFRP1 expression and clinicopathologicvariables of pancreatic pancreatic adenocarcinoma patients

Variables	SFRP1 expression		*P*-value*
	High expression	Low expression	
Age (years)			0.975
≤ 65	16	41	
> 65	10	23	
Gender			0.329
Male	14	45	
Female	12	19	
Differentiation			0.243
Well/moderate	21	40	
Poor	5	24	
pT classification			0.036
pT1	3	1	
pT2	21	48	
pT3	2	15	
Lymph node			0.081
pN0	19	34	
pN1	7	30	
Smoking			0.103
Yes (> 40 pack-years)	7	33	
No	19	31	
Perineural invasion			0.463
Yes	21	47	
No	5	17	

**P*-value were two-tailed and based on the Chi-square test or Fisher exact test.

0.001) (**Figure 3**). By univariate analysis, lymph node metastasis status, tumor differentiation and SFRP1 low expression were prognostic factors for OS. Moreover, in this study, multivariate analysis revealed that positive lymph node metastasis (P = 0.027; hazard ratio, 1.850, 95% CI, 1.074-3.185), well-moderate tumor differentiation (P = 0.030; hazard ratio, 0.547, 95% CI, 0.317-0.945), decreased SFRP1 expression (P = 0.035; hazard ratio, 0.441, 95% CI, 0.206-0.942) were 3 independent prognostic predictors for pancreatic cancer patients (**Table 2**).

Discussion

The initiation, development and metastasis of pancreatic cancer is a multi-factor, multi-step and multiple genes involved complex process [22]. Gene mutation, activation of proto-oncogene, inactivation of tumor suppressor gene and the resulting abnormal changes of signal pathways run throughout the whole process of pancreatic cancer [23]. The canonical Wnt signaling pathway takes important roles in embryonic development, tumor cell proliferation, differentiation, invasion and metastasis [24]. SFRPs are able to bind Wnt proteins in the extracellular compartment, thereby inhibiting ligand-receptor interaction and signal transduction [7]. SFRP1, a member of a family of SFRPs proteins, has been reported to involve in multiple processes during tumor development. It has been reported that SFRP1 expression is down-regulated in ovarian cancer [13], colorectal cancer [25], renal carcinoma [26]. However, the expression level of SFRP1 is little known in pancreatic cancer.

In this study, we characterized mRNA and protein expression level of SFRP1 in primary tumorous and surgical margin tissues using qRT-PCR

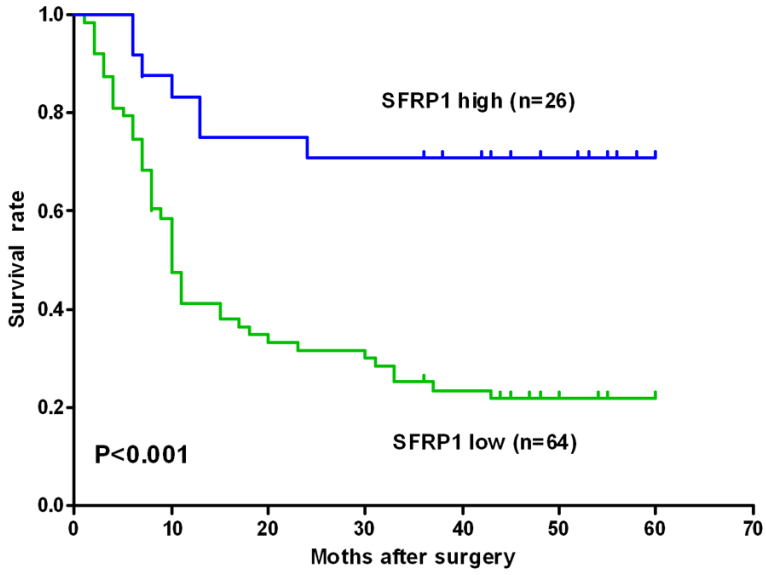

Figure 3. Kaplan-Meier analysis of the clinical outcome according to the level of SFRP1 expression. Schematic representation shows that patients with low expression of SFRP1 had a worse survival than those with high expression. P < 0.001, log-rank test.

Table 2. Univariate and multivariate analyses of factors associated with survival

Variables	HR (95% CI)	*P*-value
Univariate analysis		
Age (≤ 65 vs. > 65)	0.984 (0.573-1.689)	0.952
Gender (male vs. female)	0.602 (0.338-1.070)	0.083
Differentiation (well/moderate vs. poor)	0.495 (0.294-0.833)	0.008
PT classification		
(pT2 vs. pT1)	0.844 (0.261-2.721)	0.776
(pT3 vs. pT1)	0.929 (0.257-3.351)	0.910
(pT3 vs. pT2)	1.229 (0.649-2.328)	0.527
Lymph node (pN1 vs. pN0)	2.052 (1.224-3.439)	0.006
Smoking (Yes vs. No)	1.478 (0.886-2.467)	0.135
SFRP1 (positive vs. negative)	0.313 (0.153-0.639)	0.001
Multivariate analysis		
Lymph node (pN1 vs. pN0)	1.850 (1.074-3.185)	0.027
Differentiation (well/moderate vs. poor)	0.547 (0.317-0.945)	0.030
SFRP1 (positive vs. negative)	0.441 (0.206-0.942)	0.035

HR, hazard ratio; CI, confidence interval; *Cox's proportional hazards regression analysis (Forward stepwise).

and IHC. It revealed that SFRP1 mRNA and protein expression was significantly lower in primary pancreatic tumor tissues than paired normal samples. Furthermore, the statistic analysis, correlation between SFRP1 expression level and clinicopathologic characteristics in pancreatic cancer, found that low expression level of SFRP1 was more frequently in patients with high pT classification (P = 0.036). A recent study which indicated that SFRP1 mRNA expression was also significantly correlated with tumor stage (P = 0.02) and lymph node metastasis status (P = 0.04) via RT-PCR of 36 primary pancreatic cancers/matched-adjacent-normal samples confimed our results [18]. In our study, we have not found that correlation between SFRP1 expression with patient's lymph node metastasis status (P = 0.081). The reason might be limitations of the present study include low representation in the study cohort, a single-center experience, nonrandomized study design or potential for selection bias. Kaur [21] et al found that restoring SFRP1 levels in hepatocellular cancer cells inhibited cell growth and induced apoptotic cell death, and Jin [27] et al found that restoring SFRP1 levels in A549 human lung adenocarcinoma cell line inhibits TGF-b1-induced epithelial-mesenchymal transition (EMT) phenotype, regarded as the progression of epithelial tumors to increase the motility and invasiveness of cancer cells. Thus, based on these finding, the precise mechanism of SFRP1 involved in the progression of pancreatic cancer is worthy of further research.

Recent evidences suggest that methylated and decreased SFRP1 have been proposed as diagnostic and poor prognostic biomarker for patients with muscle-invasive bladder cancer [28-30], colorectal cancers [31], breast cancer [32], biliary tract carcinoma [33] and so on.

Conversely, high levels of SFRP1 is regarded as a biomarker for aggressive subgroups of human gastric cancer and a prognostic biomarker for patients with poor survival in gastric carcinoma [34]. In this study, we analyzed SFRP1 levels expression for 90 patients with pancreatic cancer survival rate. By univariate analysis, patients with SFRP1 expression down-regulated had a significantly poor survival rate. Furthermore, by multivariate analysis, SFRP1 expression predicted as an independent prognostic factor in pancreatic cancer using Cox proportional hazard regression model. In addition, we also found that tumor differentiation, lymph node metastasis were risk factors of survival in patients with pancreatic cancer.

In conclusion, the present study shows that SFRP1 expression in primary pancreatic tumor tissue is down-regulated. Moreover, SFRP1 expression levels is associated with clinicopathologic features and prognosis of patients with pancreatic cancer. Our findings suggest that SFRP1 low expression might play a role in promoting pancreatic cancer development and would be a prognostic biomarker in patients with pancreatic cancer.

Acknowledgements

This work was partly supported by grants from National Nature Science Foundation of China.

Disclosure of conflict of interest

None.

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