Am J Cancer Res 2016;6(11):2679-2689
www.ajcr.us /ISSN:2156-6976/ajcr0041271

Original Article

Low expression of RBMS3 and SFRP1 are associated with poor prognosis in patients with gastric cancer

Tao Zhang, Youliang Wu, Zheng Fang, Qiang Yan, Shangxin Zhang, Ruochuan Sun, Junaid khaliq, Yongxiang Li

Department of General Surgery, First Affiliated Hospital of Anhui Medical University, Hefei 230000, People's Republic of China

Received October 6, 2016; Accepted October 12, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: RNA binding motif, single stranded interacting protein 3 (RBMS3) has been reported as a tumor suppressor gene (TSG) in some squamous carcinoma. However, its expression levels and clinical significance in gastric cancer (GC) remains unclear. Secreted frizzled-related protein 1 (SFRP1) plays a role of tumor suppressor in many cancers by inhibiting Wnt/β-catenin pathway. Nevertheless, its expression levels and clinical significance in GC are in dispute. In this study, quantitative real-time PCR and Western Blot were used to measure the mRNA and protein level of RBMS3 and SFRP1 in 23 fresh GC and corresponding normal tissues. Immunohistochemistry assay was performed to further measure the protein level of RBMS3 and SFRP1 on population-based tissue microarrays consisting of 172 GC cases. We found that 69.57% (16/23) and 73.91% (17/23) GC tissues expressed remarkably lower RBMS3 than the matched normal tissues respectively in mRNA and protein levels. Similarly, 78.26% (18/23) and 65.22% (15/23) GC tissues expressed lower SFRP1 than the matched normal tissues respectively in mRNA and protein levels. Additionally, the low expression of RBMS3 and SFRP1 protein were all significantly related to the poor histological grades and prognosis (all $P<0.05$). In multivariate analysis, RBMS3 and SFRP1 co-expression status was independent prognostic factor for GC patients. Finally, the positive correlation between expression levels (mRNA and protein) of RBMS3 and SFRP1 was observed. Overall, RBMS3 and SFRP1 are both aberrantly low expressed in GC, and RBMS3 and SFRP1 co-expression is a potential prognosis predictor of GC.

Keywords: Gastric cancer, RBMS3, SFRP1, co-expression, prognosis, biomarker

Introduction

GC is one of the leading causes of global cancer-related deaths annually. There were about 951,600 new cases and 723,100 deaths due to GC worldwide per year [1]. East Asia accounting for more than half of the annual cases [2]. Although therapeutic strategies have gradually advanced recently, the prognosis of GC patients remains unsatisfactory, with a 5-year survival rate of less than 30% [3]. The main reason for such low survival is that GC is usually not diagnosed until an advanced stage. However, current chemotherapy-based treatments for patient with advanced GC do not have a significant curative effect [4]. The occurrence and development of gastric cancer is complex process involving multi-factor, multigene and multi-step. Therefore, it is essential to explore the molecular mechanism of GC and development a new bio-markers and effective therapeutic targets to improve the diagnostic sensitivity and curative effect of GC.

Various solid tumors are found that loss of chromosome 3p in recent years, suggesting that chromosome 3p may exist one or more tumor suppressor genes (TSGs) [5]. RBMS3 (RNA binding motif, single stranded interacting protein 3), a member of myc single-strand binding proteins (MSSPs) family, located at 3p24-p23 and encoded RNA-binding protein. In mammals, the RBMS family consists of 3 members termed RBMS1, RBMS2 and RBMS3. MSSPs contain two pairs of RNA binding motifs (RNP1 and RNP2) which are absolutely required for binding to the c-Myc promoter [6] and binding/stabilizing RNA in vitro [7, 8]. MSSPs involved in regulate DNA replication, transcription, apopotosis and cell cycle progression by cooperating with

the c-Myc protein [9]. The RBMS3 protein mostly expressed in the cytoplasm, suggesting it may be involved in RNA metabolism, rather than transcription [10]. The first reported target mRNA that RBMS3 binds is Prx1 mRNA. RBMS3 binds the conserved element in the 3' UTR of Prx1 mRNA leading to the up-regulation of Prx1 in hepatic stellate cells (HSCs) [8]. Recent studies shown RBMS3 have significant roles in tumor inhibition in nasopharyngeal cancer (NPC) [10], esophageal cancer [11], lung cancer and other squamous cell carcinomas [12]. It is reported that RBMS3 play a role of tumor suppressor by down-regulating c-Myc and β-catenin [6, 10]. However, the detailed function of RBMS3 in GC and its significance for clinical diagnose still remain unclear.

Secreted frizzled-related protein (SFRP) family, which has 5 members (SFRP1-5). SFRPs, as Wnt antagonists, located in a chromosomal region (8p12-p11.1). The aberrant activation of the Wnt/β-catenin signaling pathway leading to transcription of multiple oncogenes, which is one of the major molecular mechanism of GC [13-15]. The combination of Wnt and frizzled membrane receptors (Fzs) could competitively inhibited by SFRPs [16], since SFRPs compete with Fzs for Wnt ligands or direct formation no-signaling complexes with Fzs [17]. It have been reported that the abnormal expression of SFRPs associated with various tumors [18, 19]. High frequency of SFRP1 loss has been observed in colorectal cancer [17], renal cell cancer [20], breast cancer [21] and GC [22]. Moreover, SFRP1 as well as other family members were identified as TSGs [23-27]. However, the expression level of SFRP1 in GC tissues and the relationship between the expression level of SFRP1 with clinicopathological factors and prognosis of patient with GC are in dispute [22, 28, 29].

It has been reported that RBMS3 and SFRP1 could play a role of tumor suppressor by down-regulating the expression of c-Myc and β-catenin [6, 10, 23, 30]. Besides, the significant positive correlation between mRNA expression of RBMS3 and SFRP1 was observed by querying the database of TCGC (The Cancer Genome Atlas).

In this study, we further investigated the expression levels of RBMS3 and SFRP1 in human GC tissues and corresponding normal tissues, then explored the possible correlations between the clinicopathological factors and overall survival with different expression statuses of RBMS3 and SFRP1 in GC patients. Finally, we analyzed the relationship between the expression levels of RBMS3 and SFRP1 and explored whether RBMS3 and SFRP1 co-expression status hold significance for GC.

Materials and methods

Patients and tissue specimens

195 surgically treated GC patients included in the study. 23 GC tissues and matched non-tumor tissues were used for qRT-PCR and WB. 172 GC and 43 normal gastric tissues were collected for immunohistochemical. All the tissues were collected from the Department of General Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, China. The 172 GC and 43 normal gastric tissues had been collected between December 2006 and May 2008. The follow-up period is from 2006 to 2013. These specimens were incorporated into tissue microarray (TMA) for immunohistochemical staining. The 23 pairs tissue specimens for qRT-PCR and WB are fresh GC tissues and corresponding normal tissues (at least 10 cm distant from the tumor edge, verified to be free of tumor) were immediately stored at -80°C until use. All patients did not receive preoperative treatment. All tumors were collected with patients' informed consent and histologically diagnosed by experienced pathologists without any information of this study. Pathological TNM staging was evaluated using the 2010 criteria of The American Joint Committee on Cancer (AJCC). The research protocols for this study were approved by the ethics committee of the hospital.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from the 23 pairs fresh specimens using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) were used for Converting mRNA to cDNA. Expression levels of RBMS3 and SFRP1 mRNA were determined by quantitative reverse transcriptase PCR using Perfectstart SYBR Green qPCR Master Mix (Omega Bio-Tek, USA). The primers used for amplification of RBMS3 and SFRP1: RBMS3

forward primer: 5'-ACAAGAGCAAGACCCAACAAA-3', reverse primer: 5'-TGTCCAAAGGGTTTCAGCATA-3'; SFRP1, forward primer: 5'-TCATG CAGTTCTTCGGCTTC-3', reverse primer: 5'-CCAACTTCAGGGGCTTCTTCTTC-3'; GAPDH as an endogenous control, GAPDH: forward primer: 5'-AGCCACATCGCTCAGACAC-3', reverse primer: 5'-GCCCAATACGACCAAATCC-3'. The amplification protocol used the following conditions: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s, with a final extension at 72°C for 30 s. The reaction ran on the Stratagene Mx3000p Sequence Detection System (Applied Agilent, USA). Each assay reaction was performed in triplicate and the values averaged to reduce the experimental error. The $2^{-\Delta Ct}$ method was used to quantify the relative expression levels of RBMS3 and SFRP1. The expression levels of their mRNA normalized using the GAPDH expression.

Protein extraction and Western blot

Total protein were extracted from 23 pairs fresh GC and its corresponding normal mucosa tissue using RIPA lysis buffer (Beyotime, China), the protein concentration was quantified using an Enhanced BCA Protein Assay Kit (Beyotime, China). Then equivalent proteins of each pair specimens were separated by SDS-PAGE on 12% polyacrylamide gels and electrotransferred to polyvinylidene fluoride membranes (Millipore, USA). After blocking in TBST (Tris-buffered saline/Tween-20 buffer) containing 5% skim milk in for 1 h at room temperature, the membranes was placed separately in TBST solution containing anti-RBMS3 antibody (1:1000; Abcam) or anti-SFRP1 antibody (1:5000; R&D Systems), overnight at 4°C. After washing three times in TBST solution, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies in TBST plus 3% skim milk powder for 1 h at room temperature. After three washing steps in TBST solution, the target protein was visualized by an enhanced chemiluminescence detection system. Quantification of band intensity was performed with the Image J software. RBMS3 and SFRP1 band intensities were normalized with β-actin signals.

Immunohistochemistry and TMA assay

GC and normal gastric mucosa tissues were formalin-fixed, paraffin-embedded, and used to construct a TMA. H&E-stained slides were screened to identify optimal intratumoral tissue for analysis. 4-μm thick sections were baked at 63°C for 1 hour and deparaffinized with xylenes, and rehydrated in graded ethanol to distilled water. Placing the sections in boiled ethylene diamine tetraacetic acid (EDTA) for 20 min to retrieval the antigen. The sections were treated by methanol containing 3% hydrogen peroxide to quench the endogenous peroxidase activity, then, we used 1% bovine serum to block the nonspecific binding. The sections were respectively incubated with anti-RBMS3 antibody (1:25, Abcam) and anti-SFRP1 antibody (1:1000; R&D Systems) at room temperature for 30 min. Then, the section was treated with secondary antibody in working solutions. In the end, slides were placed on an autostainer link instrument and proceed with staining. The intensity of immunostaining was graded strong (+++ or 3), moderate (++ or 2), weak (+ or 1), or absent (- or 0). Tissue section was scored as the percentage of positive cells in the section (1, <10%; 2, 11-35%; 3, 36-70%; 4, >70%) [30], The multiplication of both parameters was used to evaluate the expression levels of RBMS3 and SFRP1 in GC tissues. The cutoff point for high and low expression levels was calculated by receive operating characteristic (ROC) curve analysis [31, 32], The cutoff point were as follows: low expression, total immunostaining score ≤4; high expression, total immunostaining score >4. Each slide was read and scored independently by two pathologists without any information about this study.

Statistical analysis

The SPSS 16.0 software (SPSS, USA) was used for all statistical analyses. The Pearson X^2 test was used to analyze the relationship between the clinicopathologic parameters and protein expression levels of RBMS3 and SFRP1. Univariate survival analysis and OS curve plotting using the Kaplan-Meier method, and the Log-Rank test was applied to analyze the significance of difference between groups. Multivariate survival analysis using the Cox proportional hazards regression model. Additionally, linear regression model was applied to analysis the relationship between the mRNA expression levels of RBMS3 and SFRP1. Spearman rank correlation model was used to analysis the relationship between the protein expression levels of RBMS3 and SFRP1. The student

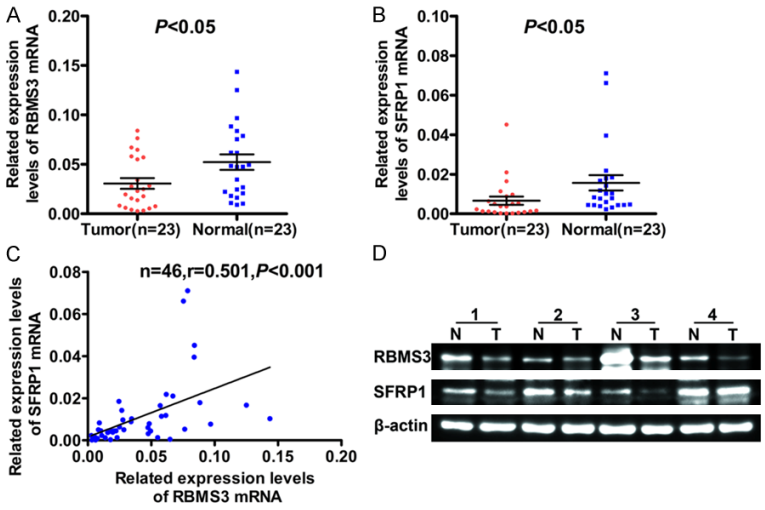

Figure 1. mRNA and protein expression levels of RBMS3 and SFRP1 in 23 GC tissues and the corresponding normal tissue. A: Relative mRNA expression of RBMS3 was detected by quantitative real-time PCR. B: Relative mRNA expression of SFRP1 was detected by quantitative real-time PCR. C: The relationship of RBMS3 and SFRP1 mRNA expression levels in 46 specimens (23 GC tissues and the corresponding normal tissue) by linear regression analysis. D: Relative protein expression of RBMS3 and SFRP1 was estimated by Western Blot assay.

t-test (two-tailed) was used to compare the differences of RBMS3 and SFRP1 expression levels between GC tissues and no-tumor tissues. P<0.05 was considered statistically significant.

Results

Expression status of RBMS3 and SFRP1 in GC

To determine the mRNA expression levels of RBMS3 and SFRP1, we performed qRT-PCR using RNA isolated from GC samples and corresponding normal gastric mucosa. As shown in **Figure 1A**, the expression levels of RBMS3 mRNA were significantly lower in 69.57% (16/23) GC tissues compared to that in corresponding normal gastric tissues. SFRP1 mRNA expression levels were significantly lower in 78.26% (18/23) GC tissues compared to paired normal tissues (**Figure 1B**). The positive correlation was observed between RBMS3 and SFRP1 mRNA expression levels (n=46, r=0.501, P<0.001) (**Figure 1C**).

Then, we verified that the protein levels of RBMS3 and SFRP1 were also markedly lower in the GC tissues than its normal controls. Using WB analysis, we demonstrated that RBMS3 and SFRP1 protein expression levels were lower in 73.91% (17/23) and 65.22% (15/23) GC tissues than the corresponding normal tissues, respectively (**Figure 1D**). The result of western blot generally conform to the outcome of qRT-PCR.

In order to further confirm this finding, we performed a tissue microarray (TMA) which consist of 172 GC tissues and 43 normal gastric tissues. Then we analyzed the immunostaining score of GC tissues. The experiment shown that the RBMS3 and SFRP1 proteins are both mainly expressed in the cytoplasm of gastric cells (**Figure 2A**). The low-expression rate of RBMS3 in GC tissues (73.26%, 126/172) is significantly higher than in normal gastric tissues (30.23%, 13/43) (P<0.05) (**Figure 2B**). Similarly, the low-expression rate of SFRP1 in GC tissues (60.47%, 104/172) is significantly higher than in normal gastric tissues (37.21%, 16/43) (P< 0.05) (**Figure 2C**). The positive correlation was observed between RBMS3 and SFRP1 protein expression (n=215, r=0.324, P<0.001) (**Figure 2D**). Based on the combined expression of RBMS3 and SFRP1, we classified the patients into four groups: RBMS3 Low/SFRP1 Low (n= 85), RBMS3 High/SFRP1 Low (n=19), RBMS3 Low/SFRP1 High (n=41) and RBMS3 High/SFRP1 High (n=27).

Relationship between RBMS3 and SFRP1 expression and clinicopathological factors of GC patients

In this study, we assessed the protein expression levels of RBMS3 and SFRP1 in the TMA. The correlation between the immunoreactivity score of RBMS3 and SFRP1 and clinical features of GC patients were analyzed (**Table 1**). Coincidentally, the protein expression levels of RBMS3 and SFRP1 were observed respectively significantly related to the histological grades (P=0.008; P=0.029), moreover, the protein expression levels of RBMS3 also related to the depth of invasion (P=0.049), but not relate to sex, age, tumor diameter, location, lymph node

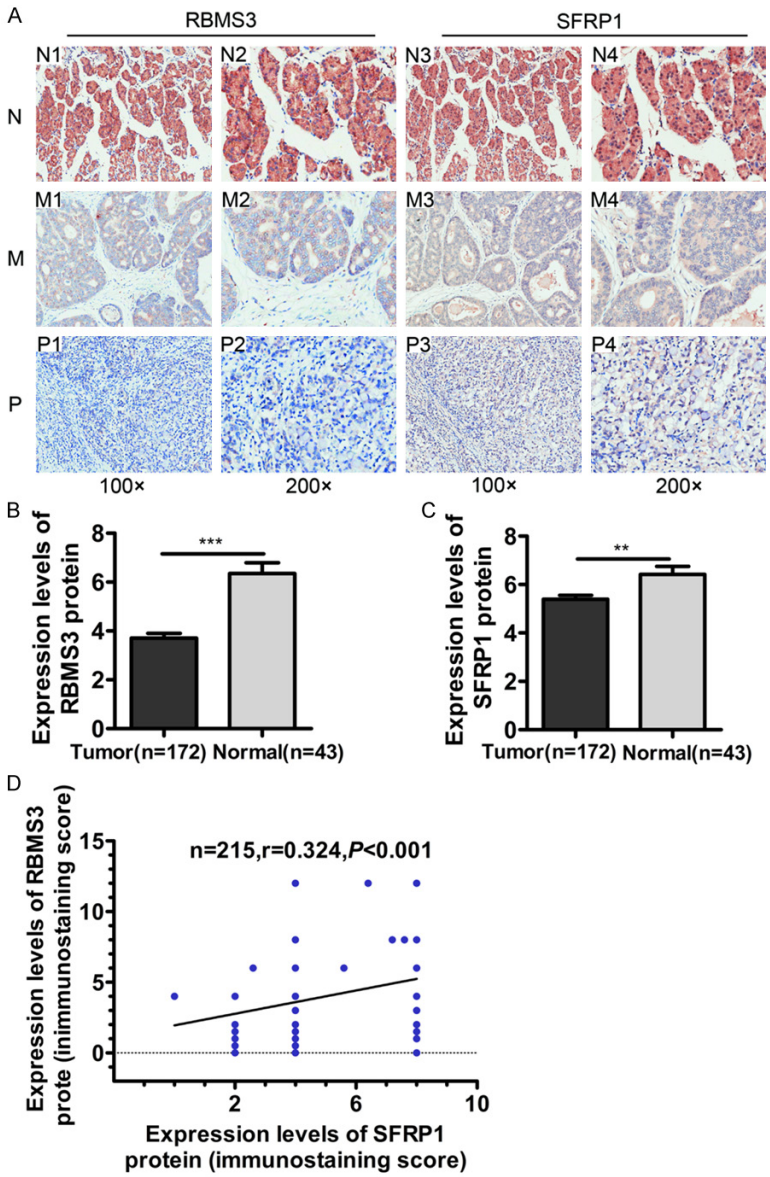

Figure 2. Immunohistochemical analysis of RBMS3 and SFRP1. A: Representative images of RBMS3 and SFRP1 expression: N1, N2, N3, N4, normal gastric tissue (N); M1, M2, M3, M4, moderately differentiated (M); P1, P2, P3, P4, poorly differentiated (P). Magnification: 100× (N1, M1, P1, N3, M3, P3), 200× (N2, M2, P2, N4, M4, P4). B: Comparison of RBMS3 protein expression levels of GC tissues (n=172) and normal gastric tissues (n=43) in TMA (***indicates P<0.001). The expression level presented as mean ± SEM. C: Comparison of SFRP1 protein expression levels of GC tissues (n=172) and normal gastric tissues (n=43) in TMA (**indicates P<0.01). The expression level presented as mean ± SEM. D: The relationship of RBMS3 and SFRP1 protein expression levels in 215 specimens (172 GC tissues and 43 normal gastric tissues) gastric tissues by spearman rank correlation analysis.

metastasis, and TNM stage (**Table 1**). Patients with poor histological grades were more likely to possess low expression of RBMS3 or SFRP1. Additionally, low expression of SFRP1 was observed more frequently in patients with deeper invasion depth of GC. We failed to discover any significance correlation between the expression levels of RBMS3 and SFRP1 and other clinicopathological factors.

Prognostic value of RBMS3 and SFRP1 expression

We use univariate Kaplan-Meier and multivariate Cox regression analyses to clarify the prognostic value of RBMS3 and SFRP1 expression. As shown in **Table 2**, we found tumor diameter, depth of invasion, histological grades, lymph node metastasis, TNM stage, RBMS3 and SFRP1 expression levels were significant associated with overall survival in univariate analysis.

The 3- and 5-year cumulative survival rates of patients with RBMS3 low expression were 62.4% and 45.8%, they were respectively lower than the 3- and 5-year cumulative survival rates (86.8% and 64.9%) of GC patients with high expression (**Table 2**). The mean survival time for GC patients with low and high expression of RBMS3 were 51.6 and 64.4 months respectively (**Table 2**). Obviously, GC patients with low expression of RBMS3 had a poorer prognosis than those with high RBMS3 expression (P<0.05) (**Figure 3A**). Similarly, The 3- and 5-year cumulative survival rates of patients with SFRP1 low expression were 65.4% and 43.7%, they were respectively lower than the 3- and 5-year cumulative survival rates of GC patients with high expression (76.3% and 62.9%) (**Table 2**). The mean survival time for GC patients with low and high expression of SFRP1 were 52.2 and 59.1 months respectively (**Table 2**). Clearly, GC patients with

Table 1. Relationships between RBMS3 and SFRP1 protein expressions (immunohistochemical staining) in GC tissues and various clinicopathological variables

Variables	Total	RBMS3 expression				SFRP1 expression			
		Low (n=126)	High (n=46)	χ^2	*P*	Low (n=104)	High (n=68)	χ^2	*P*
Gender									
Male	130	94	36	0.244	0.621	79	51	0.021	0.886
Female	42	32	10			25	17		
Age at surgery(yeas)									
<61	86	60	26	1.068	0.301	55	31	0.876	0.349
≥61	86	66	20			49	37		
Tumor diameter(cm)									
<6	112	79	33	1.213	0.271	69	43	0.175	0.676
≥6	60	47	13			35	25		
Location of primary tumor									
Cardia	83	64	19	1.215	0.269	47	36	0.989	0.320
Others	83	62	27			57	32		
Depth of invasion									
T1/T2	32	19	13	3.866	0.049	18	14	0.292	0.589
T3/T4	140	107	33			86	54		
Histological grade									
Well/moderate	52	31	21	7.078	0.008	25	27	4.785	0.029
Poor/not	120	95	25			79	41		
Lymph node metastasis									
Absent	52	38	14	0.001	0.972	32	20	0.036	0.850
Present	120	88	32			72	48		
TNM stage									
I-II	63	44	19	0.592	0.442	35	28	1.002	0.317
III-IV	109	82	27			69	40		

low expression of SFRP1 possess shorter overall survival (P<0.05) (**Figure 3B**).

For the prognostic value of the combined RBMS3 Low/SFRP1 Low expression. We found that the 3- and 5-year cumulative survival rates of patients with RBMS3 Low/SFRP1 Low expression were lower than RBMS3 High and/or SFRP1 High patients groups respectively (3-year, 60.0% vs. 79.2%; 5-year, 41.1% vs. 61.2%) (**Table 2**). The mean survival time for GC patients with RBMS3 Low/SFRP1 Low expression was shorter than those for patients with RBMS3 High and/or SFRP1 High expression (49.8 vs. 59.5 months) (**Table 2**), suggesting that RBMS3 Low/SFRP1 Low patients also had a poorer prognosis than the rest of the patients (P<0.05) (**Figure 4A**). No significant overall survival difference was observed among the patients with RBMS3 High and/or SFRP1 High expression (P=0.226), suggesting the high expression of RBMS3 or SFRP1 might functionally compensate each other's low expression in prognosis (**Figure 4B**).

We further used multivariate Cox regression analyses to elucidate the prognostic value of tumor diameter, depth of invasion, histological grades, lymph node metastasis, TNM stage, RBMS3 and SFRP1 expression. The results shown that depth of invasion (HR=5.350, 95% CI for HR 1.732-16.520, P<0.05), histological grades (HR=3.090, 95% CI for HR 1.662-5.744, P<0.05) as well as the co-expression status of RBMS3 and SFRP1 (HR=1.936, 95% CI for HR 1.240-3.021, P<0.05) were independent prognostic factors for GC patients (**Table 3**).

Discussion

This study demonstrated that both the mRNA and protein expression levels of RBMS3 and SFRP1 were lower in most GC tissues. Additionally, the low expression of RBMS3 and

Table 2. Univariate analysis of the correlation between clinicopathological parameters and survival of patients with GC

Clinicopathological Parameters	Cumulative Survival Rates, %		Mean Survival Time, month	Log-Rank Test	*P*
	3-Year	5-Year			
Gender					
Male	68.2	48.3	55.0	0.678	0.407
Female	73.8	56.3	52.9		
Age at surgery (years)					
<61	73.1	53.7	55.2	0.282	0.595
≥61	66.1	48.3	54.3		
Tumor diameter (cm)					
<6	75.0	58.5	57.1	7.110	0.008
≥6	59.4	34.7	48.9		
Location of primary tumor					
Cardia	64.9	46.7	53.9	0.691	0.406
Others	74.0	52.3	55.0		
Depth of invasion					
T1/T2	93.6	86.4	70.1	19.718	0.000
T3/T4	63.3	41.7	50.9		
Histological grade					
Well/moderate	86.5	76.4	70.0	20.076	0.000
Poor/not	62.2	39.6	48.0		
Lymph node metastasis					
Absent	90.4	74.3	68.0	18.535	0.000
Present	60.6	38.4	49.1		
TNM stage					
I-II	88.9	75.4	65.8	24.127	0.000
III-IV	58.4	34.9	47.2		
RBMS3 expression					
Low	62.4	45.8	51.6	7.069	0.008
High	86.8	64.9	64.4		
SFRP1 expression					
Low	65.4	43.7	52.2	5.340	0.021
High	76.3	62.9	59.1		
RBMS3/SFRP1 expression					
RBMS3 Low/SFRP1 Low	60.0	41.1	49.8	7.708	0.005
RBMS3 High and/or SFRP High	79.2	61.2	59.5		
RBMS3/SFRP1 expression					
RBMS3 Low/SFRP1 Low	60.0	41.1	49.8	9.889	0.020
RBMS3 High/SFRP1 Low	84.2	56.4	59.5		
RBMS3 Low/SFRP1 High	67.8	53.2	51.0		
RBMS3 High/SFRP1 High	88.9	71.5	67.0		
RBMS3/SFRP1 expression					
RBMS3 High/SFRP1 High	88.9	71.5	67.0	2.973	0.226
RBMS3 High/SFRP1 Low	84.2	56.4	59.5		
RBMS3 Low/SFRP1 High	67.8	53.2	51.0		

SFRP1 protein were respectively significantly related to the poor histological grades, and low expression of RBMS3 related to the depth of invasion. Furthermore, GC patients with low

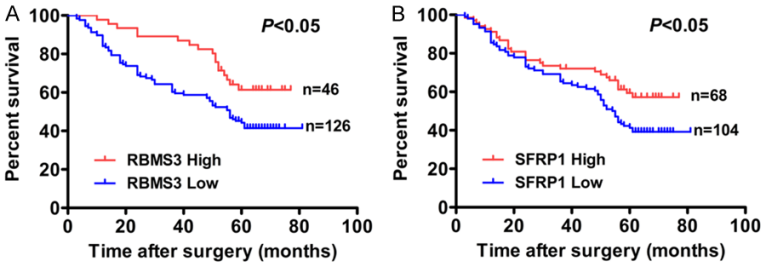

Figure 3. Kaplan-Meier analysis of the correlation between the expression of (A) RBMS3 and (B) SFRP1 and the overall survival of GC patients.

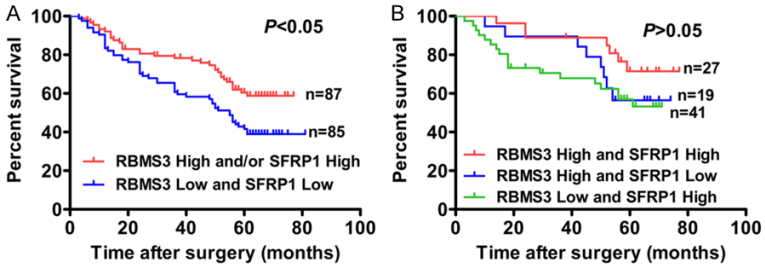

Figure 4. Kaplan-Meier analysis of the correlation between the combined RBMS3 and SFRP1 expression and the overall survival of GC patients. A: Patients with low expression of both RBMS3 and SFRP1 were compared with the rest of the patients. B: Patients with RBMS3 high and/or SFRP1 high expression were subject to analysis.

expression of RBMS3 and SFRP1 respectively had a poorer prognosis than those with high RBMS3 and SFRP1 expression. In multivariate analysis, RBMS3 and SFRP1 co-expression status was independent prognostic factor for GC patients. Finally, the positive correlation between expression levels of RBMS3 and SFRP1 was observed. This work is a prerequisite for further functional studies, it may provide a promising and useful simple bio-marker for predicting clinical outcome for GC patients.

Low expression of RBMS3 was detected in most GC tissues, and associated with poor histological grades and deep depth of invasion of GC, suggesting that the low expression of RBMS3 indicates the high malignance of GC. RBMS3, as a TSG has been confirmed in some squamous cell carcinomas [12]. c-Myc as a proto-oncogene plays an important role in tumorigenesis in many cancers. c-Myc could promote cell proliferation by stimulating expression of cyclin A, D, E, CDK2, and CDK4 [33, 34]. RBMS3 could inhibit the transcription activity of c-Myc by binding to the putative DNA replication origin sequence of the c-Myc gene in esophageal squamous cell carcinoma [6]. In addition, it has been reported that RBMS3 can inhibit the nuclear translocation of β-catenin by intercepting the expression of β-catenin in NPC cells. The translocation of β-catenin into the nucleus can initiate carcinogenesis when Wnt is present [35]. Aberrant activation of the Wnt/β-catenin signaling pathway is believed to play an important role in the tumorigenesis including GC. RBMS3 may act as tumor suppressor by inhibiting Wnt signaling pathway. Combining this experimental result, RBMS3 may act as a TSG in GC.

This study demonstrated that SFRP1 is down-regulated in most GC tissues. Additionally, low expression of SFRP1 associated with poor histological grades of GC, suggesting that the low expression of SFRP1 indicates the high malignance of GC. SFRP1 is known as Wnt antagonist, encodes a secreted glycoprotein which can competitively inhibited the combination of Wnt and Fzs receptors. Down-regulation of SFRP1 and aberrant activation of the Wnt/β-catenin signaling pathway were observed in many common malignanttumo such as NPC [30], colorectal cancer [36] and GC [37]. Downregulation of SFRP1 in many tumors mainly due to hypermethylation of the SFRP1 promoter region [25, 26]. It is reported that SFRP1 overexpression suppressed NPC cell proliferation, migration and invasion in vitro [30]. Combining with our study, SFRP1 may play a role of tumor suppressor in GC, however, these results are contrary to the previous report that SFRP1 may promote gastric tumorigenesis by activating TGFβ signaling pathway [29]. In spite of this, we still can conclude that SFRP1 may act as TSG in GC.

In this study, we further analyze the relationship between the expression levels of RBMS3 and SFRP1 in GC. Firstly, we found that both the mRNA and protein expression levels of RBMS3 and SFRP1 were significantly lower in GC tissues compared to that in corresponding nor-

Table 3. Multivariate analysis of the correlation between clinicopathological parameters and survival time of patients with GC

Covariates	Coefficient	Standard Error	HR	95% CI for HR	*P*
Tumor diameter (≥6 vs. <6)	-0.335	0.232	0.716	0.454-1.127	0.149
Depth of invasion (T3, T4 vs. T1, T2)	1.677	0.575	5.350	1.732-16.520	0.004
Histological grade (Poor/not vs. Well/moderate)	1.128	0.316	3.090	1.662-5.744	0.000
Lymph node metastasis (Present vs. Absent)	0.866	0.498	2.377	0.896-6.310	0.082
TNM stage (III-IV vs. I-II)	0.020	0.504	1.021	0.380-2.739	0.968
RBMS3/SFRP1 expression (RBMS3 Low/SFRP1 Low vs. RBMS3 High and/or SFRP1 High)	0.661	0.227	1.936	1.240-3.021	0.004

mal gastric tissues. The positive correlation between expression levels of RBMS3 and SFRP1 was observed. Secondly, low expression of them associated with poor histological grades of GC. Thirdly, RBMS3 and SFRP1 co-expression status were independent prognostic factors for GC patients. It is reasonable to believe that there is a link between RBMS3 and SFRP1 co-expression in DC. The previous studies have been reported that RBMS3 and SFRP1 could play a role of tumor suppressor by down-regulating the expression of c-Myc and β-catenin. Combining with this research results, there may be a synergistic effect between RBMS3 and SFRP1 on tumor suppression.

There are some limitations of this study. Firstly, although, the prognostic value of RBMS3 and SFRP1 co-expression for patients with GC has been demonstrated, the specific function and molecular mechanism of RBMS3 and SFRP1 in GC need further research base on in vitro studies. Secondly, in this study, the expression levels and prognostic value of SFRP1 in GC are different from the previous research results, it may result from sampling bias including representative error and registration error. A larger sample size is needed to get a more persuasive conclusion.

In summary, RBMS3 and SFRP1 co-expression status were independent prognostic factors for overall survival in GC patients. They may act as TSG, and provide a potential diagnosis and prognostic factor for GC.

Acknowledgements

We thank all the subjects of this study for their participation. This work was supported by grants from the National Natural Science Foundation of China (30871207 and 81672389) and Natural Science Research Project of Anhui Province (KJ2016A329).

Disclosure of conflict of interest

None.

Address correspondence to: Yongxiang Li, Department of General Surgery, First Affiliated Hospital of Anhui Medical University, Hefei 230000, People's Republic of China. Tel: +86-0551-62923887; E-mail: yongxiangli_1@hotmail.com

References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[2] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[3] Oba K, Paoletti X, Alberts S, Bang YJ, Benedetti J, Bleiberg H, Catalano P, Lordick F, Michiels S, Morita S, Ohashi Y, Pignon JP, Rougier P, Sasako M, Sakamoto J, Sargent D, Shitara K, Cutsem EV, Buyse M, Burzykowski T. Disease-free survival as a surrogate for overall survival in adjuvant trials of gastric cancer: a meta-analysis. J Natl Cancer Inst 2013; 105: 1600-1607.

[4] Bang YJ, Kim YW, Yang HK, Chung HC, Park YK, Lee KH, Lee KW, Kim YH, Noh SI, Cho JY, Mok YJ, Kim YH, Ji J, Yeh TS, Button P, Sirzén F, Noh SH. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): a phase 3 open-label, randomised controlled trial. Lancet 2012; 379: 315-321.

[5] Hesson LB, Cooper WN, Latif F. Evaluation of the 3p21.3 tumor-suppressor gene cluster. Oncogene 2007; 26: 7283-7301.

[6] Li Y, Chen L, Nie CJ, Zeng TT, Liu H, Mao X, Qin Y, Zhu YH, Fu L, Guan XY. Downregulation of RBMS3 is associated with poor prognosis in esophageal squamous cell carcinoma. Cancer Res 2011; 71: 6106-6115.

[7] Niki T, Izumi S, Saëgusa Y, Taira T, Takai T, Iguchi-Ariga SM, Ariga H. MSSP promotes ras/

myc cooperative cell transforming activity by binding to c-Myc. Genes Cells 2000; 5: 127-141.
[8] Niki T, Izumi S, Saëgusa Y, Taira T, Takai T, Iguchi-Ariga SM, Ariga H. Rbms3, an RNA-binding protein, mediates the expression of Ptf1a by binding to its 3'UTR during mouse pancreas development. DNA Cell Biol 2012; 31: 1245-1251.
[9] Penkov D, Ni R, Else C, Piñol-Roma S, Ramirez F, Tanaka S. Cloning of a human gene closely related to the genes coding for the c-myc single-strand binding proteins. Gene 2000; 243: 27-36.
[10] Chen J, Kwong DL, Zhu CL, Chen LL, Dong SS, Zhang LY, Tian J, Qi CB, Cao TT, Wong AM, Kong KL, Li Y, Liu M, Fu L, Guan XY. RBMS3 at 3p24 inhibits nasopharyngeal carcinoma development via inhibiting cell proliferation, angiogenesis, and inducing apoptosis. PLoS One 2012; 7: e44636.
[11] Li Y, Chen L, Nie CJ, Zeng TT, Liu H, Mao X, Qin Y, Zhu YH, Fu L, Guan XY. Downregulation of RBMS3 is associated with poor prognosis in esophageal squamous cell carcinoma. Cancer Res 2011; 71: 6106-6115.
[12] Liang YN, Liu Y, Meng Q, Li X, Wang F, Yao G, Wang L, Fu S, Tong D. RBMS3 is a tumor suppressor gene that acts as a favorable prognostic marker in lung squamous cell carcinoma. Med Oncol 2015; 32: 459.
[13] Song X, Xin N, Wang W, Zhao C. Wnt/beta-catenin, an oncogenic pathway targeted by H. pylori in gastric carcinogenesis. Oncotarget 2015; 6: 35579-88.
[14] Cheng L, Yang S, Yang Y, Zhang W, Xiao H, Gao H, Deng X, Zhang Q. Global gene expression and functional network analysis of gastric cancer identify extended pathway maps and GPRC5A as a potential biomarker. Cancer Lett 2012; 326: 105-13.
[15] Ara H, Takagishi M, Enomoto A, Asai M, Ushida K, Asai N, Shimoyama Y, Kaibuchi K, Kodera Y, Takahashi M. Role for Daple in non-canonical Wnt signaling during gastric cancer invasion and metastasis. Cancer Sci 2016; 107: 133-139.
[16] Jones SE, Jomary C. Secreted Frizzled-related proteins: searching for relationships and patterns. Bioessays 2002; 24: 811-820.
[17] Caldwell GM, Jones C, Gensberg K, Jan S, Hardy RG, Byrd P, Chughtai S, Wallis Y, Matthews GM, Morton DG. The Wnt antagonist sFRP1 in colorectal tumorigenesis. Cancer Res 2004; 64: 883-888.
[18] Ko J, Ryu KS, Lee YH, Na DS, Kim YS, Oh YM, Kim IS, Kim JW. Human secreted frizzled-related protein is down-regulated and induces apoptosis in human cervical cancer. Exp Cell Res 2002; 280: 280-287.
[19] Caldwell GM, Jones CE, Taniere P, Warrack R, Soon Y, Matthews GM, Morton DG. The Wnt antagonist sFRP1 is downregulated in premalignant large bowel adenomas. Br J Cancer 2006; 94: 922-927.
[20] Dahl E, Wiesmann F, Woenckhaus M, Stoehr R, Wild PJ, Veeck J, Knüchel R, Klopocki E, Sauter G, Simon R, Wieland WF, Walter B, Denzinger S, Hartmann A, Hammerschmied CG. Frequent loss of SFRP1 expression in multiple human solid tumors: association with aberrant promoter methylation in renal cell carcinoma. Oncogene 2007; 26: 5680-5691.
[21] Suzuki H, Toyota M, Carraway H, Gabrielson E, Ohmura T, Fujikane T, Nishikawa N, Sogabe Y, Nojima M, Sonoda T. Frequent epigenetic inactivation of Wnt antagonist genes in breast cancer. Br J Cancer 2008; 98: 1147-1156.
[22] Kinoshita T, Nomoto S, Kodera Y, Koike M, Fujiwara M, Nakao A. Decreased expression and aberrant hypermethylation of the SFRP genes in human gastric cancer. Hepatogastroenterology 2011; 58: 1051-1056.
[23] Zheng L, Sun D, Fan W, Zhang Z, Li Q, Jiang T. Diagnostic Value of SFRP1 as a Favorable Predictive and Prognostic Biomarker in Patients with Prostate Cancer. PLoS One 2015; 10: e0118276.
[24] Luo X, Wei B, Chen A, Zhao H, Huang K, Chen J. Methylation-mediated loss of SFRP2 enhances melanoma cell invasion via Wnt signaling. Am J Transl Res 2016; 8: 1502-1509.
[25] Ekström EJ, Sherwood V, Andersson T. Methylation and loss of Secreted Frizzled-Related Protein 3 enhances melanoma cell migration and invasion. PLoS One 2011; 6: e18674.
[26] Bhuvanalakshmi G, Arfuso F, Millward M, Dharmarajan A, Warrier S. Secreted Frizzled-Related Protein 4 Inhibits Glioma Stem-Like Cells by Reversing Epithelial to Mesenchymal Transition, Inducing Apoptosis and Decreasing Cancer Stem Cell Properties. PLoS One 2015; 10: e0127517.
[27] Kawakami K, Yamamura S, Hirata H, Ueno K, Saini S, Majid S, Tanaka Y, Kawamoto K, Enokida H, Nakagawa M, Dahiya R. Secreted frizzled-related protein-5 is epigenetically downregulated and functions as a tumor suppressor in kidney cancer. Int J Cancer 2011; 128: 541-550.
[28] Zhao CH, Bu XM, Zhang N. Hypermethylation and aberrant expression of Wnt antagonist secreted frizzled-related protein 1 in gastric cancer. World J Gastroenterol 2007; 13: 2214-2217.
[29] Qu Y, Ray PS, Li J, Cai Q, Bagaria SP, Moran C, Sim MS, Zhang J, Turner RR, Zhu Z, Cui X, Liu B. High levels of secreted frizzled-related protein 1 correlate with poor prognosis and

promote tumorigenesis in gastric cancer. Eur J Cancer 2013; 49: 3718-3728.
[30] Ren XY, Zhou GQ, Jiang W, Sun Y, Xu YF, Li YQ, Tang XR, Wen X, He QM, Yang XJ, Liu N, Ma J. Low SFRP1 expression correlates with poor prognosis and promotes cell invasion by activating the Wnt/β-catenin signaling pathway in NPC. Cancer Prev Res (Phila) 2015; 8: 968-977.
[31] Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 1982; 143: 29-36.
[32] Zweig MH, Campbell G. Receiver operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993; 39: 561-577.
[33] Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999; 13: 1501-1512.
[34] Beier R, Bürgin A, Kiermaier A, Fero M, Karsunky H, Saffrich R, Möröy T, Ansorge W, Roberts J, Eilers M. Induction of cyclin E-cdk2 kinase activity, E2F-dependent transcription and cell growth by Myc are genetically separable events. EMBO J 2000; 19: 5813-5823.
[35] Taurin S, Sandbo N, Qin Y, Browning D, Dulin NO. Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase. J Biol Chem 2006; 281: 9971-9976.
[36] Silva AL, Dawson SN, Arends MJ, Guttula K, Hall N, Cameron EA, Huang TH, Brenton JD, Tavaré S, Bienz M, Ibrahim AE. Boosting Wnt activity during colorectal cancer progression through selective hypermethylation of Wnt signaling antagonists. BMC Cancer 2014; 14: 891.
[37] Yoda Y, Takeshima H, Niwa T, Kim JG, Ando T, Kushima R, Sugiyama T, Katai H, Noshiro H, Ushijima T. Integrated analysis of cancer-related pathways affected by genetic and epigenetic alterations in gastric cancer. Gastric Cancer 2015; 18: 65-76.