Original Paper

Lower Expression of SPRY4 Predicts a Poor Prognosis and Regulates Cell Proliferation in Colorectal Cancer

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Key Words

Methylation • SPRY4 • Colorectal cancer • Cell proliferation

Abstract

Background/Aims: Colorectal cancer (CRC) is the third most common type of cancer worldwide. Sprouty proteins are modulators of mitogen-induced signal transduction processes and therefore can influence the process of cancerogenesis. The encoded protein of Sprouty homolog 4 (SPRY4) is associated with various human cancers. However, its biological role and clinical significance in CRC development and progression are unknown. **Methods:** The aim of this study was to evaluate the expression and biological role of SPRY4 in colorectal cancer. qRT-PCR was performed to investigate the expression of SPRY4 in tumor tissues and corresponding non tumor colorectal tissues from 70 patients. The effect of SPRY4 on proliferation was evaluated by MTT and colony formation assays. CRC cells transfected with SPRY4 were injected into nude mice to study the effect of SPRY4 on tumorigenesis *in vivo*. **Results:** The lower expression of SPRY4 was remarkably correlated with deep tumor invasion and advanced TNM stage. Multivariate analyses revealed that SPRY4 expression served as an independent predictor for overall survival. Using 5-aza treatment, we also observed that SPRY4 expression can be affected by DNA methylation. Further experiments revealed that overexpressed SPRY4 significantly inhibited CRC cell proliferation both *in vitro* and *in vivo*. **Conclusion:** Our study demonstrated that SPRY4 is involved in the development and progression of colorectal cancer by regulating cell proliferation and shows that SPRY4 may be a potential diagnostic and prognostic target in patients with colorectal cancer.

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Introduction

Colorectal cancer (CRC) is an important public health problem affecting over a million people, and it is the third most common type of cancer worldwide [1-3]. Due to chemotherapy and radiation therapy, the incidence and mortality of CRC have decreased in recent years. However, the five-year survival rate of CRC patients remains relatively low. It is essential to find new biomarkers for metastatic progression in colorectal cancer.

Sprouty (Spry) proteins are known modulators of RTK-mediated signaling inhibiting pathways downstream of growth factors [4]. In humans, four Spry protein family members have been discovered [5]. Sprouty proteins are modulators of mitogen-induced signal transduction processes and therefore can influence the process of carcinogenesis. Here, we focus on SPRY4. SPRY4 is located in 5q31 and encodes a member of a family of cysteine- and proline-rich proteins. Wang et al. reported that SPRY4 can serve as a suppressor of tumor cell motility and is down regulated by DNA methylation in human prostate cancer [6]. In addition, in human breast cancer, ectopic SPRY4 expression can inhibit cell proliferation and migration of breast cancer cell lines [7]. SPRY4 can also inhibit cell proliferation and migration in non-small cell lung cancer [8]. These results indicate that the dysregulation of SPRY4 may participate in human cancer progression. However, the biological functions of SPRY4 in the control of CRC tumorigenesis have not been well characterized, which prompted us to explore the role of SPRY4 in human CRC.

In this study, we found that SPRY4 expression was decreased in colorectal cancer tissues. The correlation between SPRY4 downregulation and clinical-pathological characteristics was also studied. The effect of DNA methylation on SPRY4 expression was investigated as well. Moreover, overexpression of SPRY4 could inhibit cellular proliferation both *in vitro* and *in vivo*. Taken together, the results of this study indicated that SPRY4 plays an important role in colorectal cancer development and could be a potential therapeutic target for patients with colorectal cancer.

Material and Methods

Tissue samples and clinical data collection

Paired colorectal cancer and adjacent non-tumor colon tissues were obtained from 70 patients who underwent primary surgical resection of colorectal cancer at the First Affiliated Hospital Guangxi University of Chinese Medicine (Nanning, Guangxi, and PR China). The clinicopathological factors of patients are shown in Table 1. The study was approved by the Research Ethics Committee of Guangxi University of Chinese Medicine, and written informed consent was obtained from all patients. Colorectal cancer and normal tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until total RNA was extracted.

Cell lines and culture conditions

Human colorectal cancer cell lines HCT-116 and Lovo were obtained from the Chinese Academy of Sciences Committee on Type Culture Collection Cell Bank. These cells were cultured in DMEM (GIBCO-BRL) medium supplemented with 10% fetal bovine serum (Invitrogen, Shanghai, China), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Shanghai, China) in an incubator at 37°C with 5% CO₂.

Treatment of HCT-116 and Lovo cells with 5-aza

HCT-116 and Lovo cells were seeded into a six-well culture plate and exposed to 10 μM 5-aza-CdR (Sigma-Aldrich, USA) for 3 days. The cells treated with 5-aza-CdR were harvested and used for detection of SPRY4 expression

Transfection of colorectal cancer cells

The sequence of SPRY4 was synthesized and subcloned into a pcDNA3.1 vector (Invitrogen, Shanghai, China). The pcDNA-SPRY4 or empty pcDNA3.1 vector was transfected into colorectal cancer cells using the X-tremeGENE HP DNA transfection reagent (Roche, Basel, and Switzerland) according to the manufacturer's

instructions. The empty pcDNA3.1 vector was used as the control. Cells were harvested 48 h after transfection.

RNA extraction and qRT-PCR analyses

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For qRT-PCR, RNA was reverse transcribed to cDNA using a PrimeScript RT reagent Kit (Takara, Dalian, China). Real-time PCR analyses were performed with SYBR Green (Takara, Dalian China). The results were normalized to the expression of GAPDH. The PCR primers for SPRY4 or GAPDH were as follows: SPRY4 forward, 5'-CTGCCCATCTACACCTCAG-3' and reverse, 5'-CTCTCCGCGCTCTGCGCTAGGGGCT-3'; GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3' and reverse, 5'-GCCCAATACGACCAATCC-3'. qRT-PCR and data collection were performed on ABI 7500. The relative expression of SPRY4 was calculated and normalized using the 2- $\Delta\Delta C_t$ method relative to GAPDH.

Cell proliferation assays

Cell proliferation Reagent Kit I (MTT) (Roche, Basel, Switzerland) was used to assess cell proliferation. Transfected cells were plated in each well of a 96-well plate and assessed every 24 h according to the manufacturer's instructions. For colony formation assay, a certain number of transfected cells were placed in each well of a six-well plate and maintained in proper medium containing 10% FBS for approximately 14 days, replacing the medium every 3 days. The colonies were then fixed with methanol and stained with 0.1% crystal violet (Sigma, USA); the colony formation was determined by counting the number of stained colonies.

Flow-cytometric analysis of cell cycle

Transfected cells were harvested after transfection by trypsinization. Cells for cell-cycle analysis were stained with propidium oxide by the Cycle TEST PLUS DNA Reagent Kit (BD Biosciences, USA) following the protocol and analyzed by FACScan. The percentage of the cells in G0-G1, S, and G2-M phase were counted and compared.

Western blot assay and antibodies

Cell protein lysates were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) transferred to 0.22- μ m NC membranes (Sigma, USA) and incubated with specific antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). GAPDH antibody was used as control. Anti-SPRY4 was from Santa Cruz. Anti-Cyclin D1 was purchased from Cell Signaling Technology (Boston, MA, USA).

Tumor formation assay in a nude mouse model

Five-week-old male athymic BALB/c mice were maintained under specific pathogen-free conditions and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. HCT-116 cells transfected with pcDNA3.1 vector or pcDNA-SPRY4 were harvested at a concentration of 2×10^7 cells/ml. Of the suspending cells, 0.1 ml was subcutaneously injected into either side of the posterior flank of the nude mouse. Tumor volumes were measured (length $\times$ width² $\times$ 0.5) every 2 days in mice from the control (three mice) or pcDNA-SPRY4 (three mice) groups. Sixteen days after injection, the mice were killed and tumor weights were measured and used for further analysis. The primary tumors were excised, and tumor tissues were used to perform immunostaining analysis of Ki-67 protein expression.

Table 1. Clinicopathological characteristics of CRC patients

Characteristics	Expression of SPRY4		p value*
	low	high	
Sex			0.47
male	21	18	
female	14	17	
Age			0.151
≤ 60	21	15	
> 60	14	20	
Histological grade			0.031*
Low	32	25	
middle or high	3	10	
Tumor invasion depth			0.022*
T1	2	9	
T2 or above	33	26	
TNM stage			0.006**
I	1	9	
II / III / IV	34	26	

Bisulfite genomic sequencing PCR (BSP)

BSP was performed in cell lines following transfection with si-NC and siRNA-LINC01116. Genomic DNA was isolated using the Genomic DNA Extraction kit (Takara Bio, Inc.) according to the manufacturer's instructions. Bisulfite modification (EZ DNA Methylation-Gold™ kit, D5005/50; Zymo Research Corp. Irvine, CA, USA) was performed to convert unmethylated cytosine to uracil; methylated cytosine nucleotides are unaffected by the procedure. Bisulfite-modified gene promoters were amplified using specially designed primers. Per sample, ten independent colonies for each tested region were picked and sequenced.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using EZ-CHIP KIT according to the manufacturer instructions (Millipore, USA). DNMT1 was obtained from Abcam. Quantification of immunoprecipitated DNA was performed using qPCR. ChIP data was calculated as a percentage relative to the input DNA using the equation $2^{[Input Ct - Target Ct]} \times 0.1 \times 100$.

Statistical analysis

Statistical analysis was performed using SPSS version 18 software (Chicago, IL). The chi-square and t tests were performed to explore the associations between the SPRY4 expression level and the clinical characteristics. The survival curves were estimated using the Kaplan–Meier method. The log-rank test was used to estimate the significance of the differences between the survival curves. A Cox proportional hazards analysis was performed to calculate the hazard ratio (HR) and the 95% confidence interval (CI) to evaluate the association between SPRY4 expression and overall survival time (OS). A multivariate Cox regression was performed to adjust for other covariates. P-values less than 0.05 are considered to be statistically significant.

Results

SPRY4 expression is downregulated in CRC tumor tissues and significantly correlated with tumor invasion, metastasis and TNM stage

First, we examined SPRY4 level in CRC tumor tissues. The expression of SPRY4 in tumor tissues relative to adjacent normal tissues is shown in Fig. 1A. Among all the 70 pairs of CRC patients, SPRY4 expression levels in tumors were lower than those in the corresponding normal tissues on the whole ($P < 0.001$) (Fig. 1A). Next, we explored the correlation of SPRY4 expression level with the clinic-pathological factors in CRC patients. The results demonstrated that deeper tumor invasion depth and advanced TNM stage was significantly positively related to decreased SPRY4 expression, with values of 0.2235 ± 0.26264 vs 1.2961 ± 1.79598 ($P = 0.044$) and 0.2251 ± 0.26452 vs 1.7159 ± 1.98417 ($P = 0.042$), respectively (Fig. 1B and 1C).

Next, we divided the samples into high (above the mean, $n = 35$) and low (below the mean, $n = 35$) SPRY4 expression groups according to the mean value of SPRY4 level. A chi-square test was also performed to examine the correlation of SPRY4 expression level with the clinic-pathological factors of patients. As a result, SPRY4 expression levels were also found to be correlated to histological grade ($P = 0.031$), tumor invasion depth ($P = 0.022$) and TNM stage ($P = 0.006$). However, patients' age (≤ 60 , > 60) and patients' sex (male, female) were not found to be significantly correlated with SPRY4 in our study (Table 1).

Univariate and multivariate analysis show SPRY4 expression is an independent predictor for overall survival

To further evaluate the value of SPRY4 in prognosis of patients with CRC, we used Kaplan-Meier survival analysis and log-rank tests. As shown in Fig. 1D, decreased SPRY4 expression levels were associated with shorter overall survival ($P < 0.001$). Univariate analysis identified five prognostic factors: histological grade (low, middle or high), TNM stage (I, II/III/IV) and SPRY4 expression. The other clinic-pathological characteristics, such as tumor invasion depth (T1, T2 or above), age (≤ 60 , > 60) and sex (male, female), were not statistically significant prognosis factors. Multivariate analysis further revealed that SPRY4

Fig. 1. Analysis of SPRY4 expression in CRC tissues and clinical parameters. (A) Relative expression of SPRY4 in CRC tissues (n = 70) compared with corresponding non-tumor tissues (n = 70). The levels of SPRY4 in CRC tissues are significantly lower than those in non-tumorous tissues. The Δ Ct value was determined by subtracting the GAPDH Ct value from the SPRY4 Ct value. Smaller Δ Ct value indicates higher expression. (B and C) SPRY4 expression was significantly lower in patients with advanced tumor invasion depth and a high TNM stage. (D) Kaplan–Meier analysis of overall survival was analyzed according to SPRY4 expression levels.

Table 2. Univariate and multivariate Cox regression analyses of SPRY4 for OS of patients in study cohort (n = 70)

Risk factors	Univariate analysis			multivariate analysis		
	HR*	p value	95% CI	HR	p value	95% CI
SPRY4 expression	0.105	0.005**	0.021~0.511	0.157	0.026*	0.031~0.805
TNM stage (I, II/III/IV)	3.506	0.017*	1.252~9.816	0.958	0.951	0.246~3.729
Histological grade (low, middle or high)	2.364	0.049*	1.003~5.570	1.506	0.443	0.530~4.281
Age (≤ 60 , > 60)	0.708	0.233	0.401~1.248			
Tumor invasion depth (T1, T2 or above)	2.12	0.067	0.950~4.729			
Sex (male, female)	0.865	0.619	0.488~1.533			

expression was a significant independent predictor of poor survival of CRC patients (P = 0.026) (Table 2).

SPRY4 is modulated by DNA methylation, and overexpression of SPRY4 inhibits colorectal cancer cell proliferation in vitro

Hypermethylation of the SPRY4 promoter region has been reported to contribute to SPRY4 transcriptional inactivation [6, 9]. To examine the role of aberrant methylation

Fig. 2. SPRY4 is modulated by DNA methylation, and overexpression of SPRY4 inhibits colorectal cancer cell proliferation *in vitro*. (A) qRT-PCR analyses of SPRY4 expression level following treatment of HCT-116 and Lovo cells with 10 μ M 5-aza. The methylation status of the promoter of SPRY4 was assessed by bisulfite sequencing in CRC and normal tissues (n = 5). Open and filled squares denote unmethylated and methylated CpG sites, respectively. Enrichment of DNMT1 in the SPRY4 promoter after DNMT1 knockdown by ChIP assays. (B) The relative expression level of SPRY4 in HCT-116 and Lovo cells, transfected with empty vector or pCDNA-SPRY4, was tested by western blot analysis. (C) At 48 h after transfection, MTT assay was performed to determine the proliferation of HCT-116 and Lovo cells. (D) Representative results of colony formation of HCT-116 and Lovo cells transfected with empty vector or pCDNA-SPRY4. *, P < 0.05; **, P < 0.01.

in the deregulation of SPRY4 in colorectal cancer cells, we evaluated the effect of a DNA demethylating agent (5-aza-CdR) on SPRY4 expression. Following treatment of HCT-116 and Lovo cells with 5-aza-CdR, we found that SPRY4 expression was significantly increased in 5-aza-treated cells compared with control cells (Fig. 2A). In addition, we performed Bisulfite genomic sequencing PCR (BSP) assays to detect the status of promoter methylation of SPRY4 in human samples. As shown in Fig. 2A, the average frequency of methylation was 44% in normal tissues and 80% in colorectal cancer tissues. These results indicate that hypermethylation of the SPRY4 promoter region contributed to SPRY4 transcriptional inactivation in CRC. In addition, as shown in Fig. 2A, chromatin immunoprecipitation (ChIP) experiments were conducted, which validated that DNMT1 (DNA methyltransferase 1) could indeed bind to the promoter region of SPRY4, and knockdown of DNMT1 could decrease the occupancy of DNMT1 in the promoter of SPRY4. These results suggest that DNMT1 could serve as a regulator of SPRY4.

We also investigated the functional role of SPRY4 in colorectal cancer cells. Forty-eight hours after transfection of a pCDNA-SPRY4 or empty plasmid, western blot analysis revealed that SPRY4 expression was obviously upregulated in HCT-116 and Lovo cell lines (Fig. 2B). Furthermore, MTT and colony formation assays were also performed. MTT assays

Fig. 3. The impact of SPRY4 on tumorigenesis *in vivo*. (A) and (B) Empty vector or pCDNA-SPRY4 was transfected into HCT-116 cells, which were injected into nude mice (n=3), respectively. Tumor volumes were measured every two days after injection. Bars indicate SD. (C) Tumor weights are represented as the means $\pm$ SD. (D) H&E and IHC staining was performed on tumor sections using antibodies against Ki-67. Error bars indicate means $\pm$ standard errors of the mean. *, $P < 0.05$; **, $P < 0.01$.

showed that overexpressed SPRY4 could inhibit cell viability in both HCT-116 and Lovo cell lines when compared with control cells (Fig. 2C). Colony-formation assays showed that the number of clones was significantly decreased in both the HCT-116/ pCDNA-SPRY4 group and the Lovo/ pCDNA-SPRY4 group (Fig. 2D).

Overexpression of SPRY4 inhibits colorectal cancer cell proliferation *in vivo*

To explore whether SPRY4 expression affects tumor growth *in vivo*, HCT-116 cells that were transfected with pCDNA-SPRY4 or an empty vector were inoculated into nude mice. At the injection site, all mice developed xenograft tumors. Tumor growth in the pCDNA-SPRY4 group was significantly slower than that in the empty vector group on the 16th day after injection (Fig. 3A and 3B). The average tumor weight in the pCDNA-SPRY4 group was lower than that in the control group (Fig. 3C). HE staining showed the typical characteristics of tumor cells (Fig. 3D). The results of immunohistochemical staining showed that the proliferation index ki-67 was significantly decreased in the SPRY4-transfected tumors (Fig. 3D). Taken together, these results demonstrated that overexpression of SPRY4 could inhibit colorectal cancer cell proliferation *in vivo*.

SPRY4 regulates proliferation of CRC cells by altering the cell cycle

Next, flow cytometric analysis was performed to further examine the effect of SPRY4 on proliferation of CRC cells by altering cell cycle progression. As shown in Fig. 4A, HCT116/ pCDNA-SPRY4 cells were significantly stalled at the G1-G0 phase compared with cells transfected with empty vector. Similar results were also observed in the Lovo cell line. Moreover, the expression levels of Cyclin D1 and Cyclin E, which are cell cycle marker proteins, were significantly decreased both in HCT-116 and Lovo cells transfected with pCDNA-SPRY4. However, after 48h, the expression of pERK and p21 remained unchanged

Fig. 4. SPRY4 regulates colorectal cancer cell proliferation by affecting cell cycle. (A) Forty-eight hours after transfection, the cell cycle of HCT-116 and Lovo was analyzed by flow cytometry. The bar chart represents the percentage of cells in G1-G0, S, or G2-M phase, as indicated. (B) Western blot analysis of Cyclin D1 and Cyclin E after pCDNA-SPRY4 or empty vector transfection of HCT-116 and Lovo cells. GAPDH protein was used as an internal control. The results shown are from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Fig. 5. Western blot analysis of pERK and p21 after pCDNA-SPRY4 or empty vector was transfected into HCT-116 cells. GAPDH protein was used as an internal control.

after overexpression of SPRY4 (Fig. 5). These data suggested that SPRY4 may function as a tumor suppressor gene partly by affecting the cell cycle in colorectal cancer.

Discussion

In our present study, we were interested in understanding the role of SPRY4 in colorectal cancer. We found that SPRY4 was downregulated in colorectal cancer tissues compared to normal tissues. Furthermore, lower expression of SPRY4 was remarkably correlated with

deep tumor invasion and advanced TNM stage. The findings of our study are consistent with previous studies, which have shown that SPRY4 was downregulated in many cancers, including prostate cancer [6], NSCLC [8], and breast cancer [7]. These observations indicate that SPRY4 may function as a tumor suppressor in human tumor progression. In earlier reports, SPRY2 was shown to play a tumor suppressive role, for example, in prostate [10], lung [11], breast [12], and liver cancer [13, 14]. Moreover, SPRY1 could serve as a tumor suppressor in many types of cancer, including breast cancer, thyroid carcinoma and epithelial ovarian cancer [15-17]. These results indicated that as modulators of signal transduction, spry proteins are capable of influencing tumorigenic transformation processes.

The prognostic role of SPRY4 in cancer remains unclear. To determine the relationship between SPRY4 expression and CRC patients' prognosis, we attempted to evaluate the correlation between SPRY4 expression and clinical outcomes. Kaplan-Meier analysis showed that patients with low levels of SPRY4 expression had remarkably shorter survival time than those with high levels. Multivariate analysis further revealed that SPRY4 expression was a significant independent predictor of poor survival of CRC patients. These results indicate that downregulation of SPRY4 may have important roles in CRC development and progression.

We performed MTT and colony formation assays *in vitro* to investigate the biological function of SPRY4 in CRC cells. Overexpression of SPRY4 correlated with lower cell viability compared to the control group both in HCT-116 and Lovo cell lines. In addition, flow-cytometric analysis showed that overexpression of SPRY4 induced arrest of cell cycle. Previous studies have consistently demonstrated that SPRY4 could inhibit cancer cell proliferation [7, 8]. Moreover, we found that DNA methylation could lead to the downregulation of SPRY4 in CRC cancer. DNA methylation can suppress SPRY gene family expression in different types of tumors, including SPRY1, SPRY2, and SPRY4 [6, 9, 13, 18, 19]. *In vivo*, our results showed that the average tumor weight or volume was significantly lower for the mice injected with SPRY4-overexpressed HCT-116 cells compared to control cells. SPRY4 overexpression can effectively suppress the progression of CRC xenografts. Previous studies have shown that the Spry protein family may regulate cell growth through the ERK MAPK pathway [12]. In addition, p21 was a major transducer of tumor suppression. We deduced that SPRY4 could function by affecting pERK and p21. As shown in Fig. 5, SPRY4 has no effect on the expression of pERK and p21. These data indicated that SPRY4 could not suppress cell proliferation this way in CRC. Our results showed that Cyclin D1 and Cyclin E, the cell cycle marker proteins, were both significantly decreased in CRC cells. Therefore, SPRY4 could function as a suppressor of proliferation partly by inducing arrest of cell cycle in CRC. These results have identified an important role for SPRY4 in CRC and clarified the potential application of SPRY4 in CRC.

In summary, to our knowledge, this is the first report showing the role of SPRY4 in colorectal cancer. Our results indicate that SPRY4 is an important indicator of poor survival rate and an independent prognostic factor for colorectal cancer patients. We also showed that SPRY4 can regulate colorectal cancer cell proliferation both *in vitro* and *in vivo*. These results suggest that SPRY4 plays an important role in colorectal cancer.

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Disclosure Statement

The authors have declared that no conflicts of interest exist.

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