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ORIGINAL ARTICLE

Hypermethylated Promoters of Secreted Frizzled-Related Protein Genes are Associated with Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of the leading causes of death worldwide. Aberrant DNA methylation has been recognized as one of the most common molecular alterations in CRC. The goal of this study was to investigate the diagnostic value of *SFRP1* and *SFRP2* methylation for CRC. A total of 80 pairs of CRC patients were recruited to test the association of *SFRP1* and *SFRP2* promotor methylation with CRC. Methylation assay was performed using quantitative methylation-specific polymerase chain reaction (qMSP) method. In this study, we found the methylation levels of *SFRP1* and *SFRP2* in CRC tumor tissues were significantly higher than those in the adjacent non-tumor tissues (*SFRP1*: $P = 2E\text{-}5$; *SFRP2*: $P = 0.014$). Further bioinformatics analysis of TCGA data confirmed the association of the two genes with CRC (*SFRP1*: $P = 7E\text{-}21$; *SFRP2*: $P = 5E\text{-}24$). Luciferase reporter gene assay showed that the recombinant plasmids with *SFRP1* and *SFRP2* fragments could significantly enhance promoter activity (*SFRP1*: $P = 0.002$; *SFRP2*: $P = 0.004$). In addition, *SFRP1* and *SFRP2* methylation were inversely correlated with the mRNA expression displayed by TCGA data mining (*SFRP1*: $r = -0.432$, $P = 4E\text{-}11$; *SFRP2*: $r = -0.478$, $P = 1E\text{-}13$). GEO data analysis indicated that *SFRP1* and *SFRP2* expression were increased in three CRC cell lines (COLO320, HCT116 and HT29) after 5′-AZA-deoxycytidine treatment, suggesting that DNA methylation played an important role in regulating gene expression of the two genes. Our results confirmed that promoter methylation of *SFRP1* and *SFRP2* contributed to the risk of CRC.

Keywords *SFRP1* · *SFRP2* · DNA methylation · Colorectal cancer · Quantitative methylation specific PCR

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide [1, 2]. Despite of the recent improvements in the therapy, CRC remains as a global public health problem due to its poor prognosis [3]. Evidence has shown that early diagnosis of CRC is vital for the outcome of CRC [4]. Thus, identification of new biomarkers with high sensitivity and specificity is necessary for the early diagnosis of CRC.

Aberrant gene methylation has been shown to contribute to the loss of gene function and the occurrence of cancers [5–9], including CRC [2, 10, 11]. Meanwhile, methylated DNA is chemically and biologically stable and less subjected to transient alterations [12], which considered as an ideal biomarker in the occurrence and progress of carcinoma.

SFRP1 and *SFRP2* are tumor suppressor genes of the *SFRP* family. Wnt proteins and Frizzled receptors can interact with each other through *SRPF1* in the extracellular compartment. It is noteworthy that *SFRP1* plays a key role in embryonic development, cell differentiation and proliferation [13]. *SFRP2* protein can inhibit Wnt receptor from binding to downregulate pathway signaling [14]. Previous studies showed that *SFRP1* and *SFRP2* hypermethylation had been found in CRC [15–20]. However, those studies were involved with a relative fewer CRC samples (from 15 to 52) in Europeans and Asians. And most of those studies were

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performed by methylation specific PCR (MSP), a qualitative approach with a low sensitivity in methylation detection [3].

In this study, *SFRP1* and *SFRP2* methylation was measured by quantitative methylation specific PCR (qMSP) in tumor and para-tumor tissues of 80 Chinese Han CRC patients. The aim of the present study was to investigate the association between candidate genes methylation and CRC.

Materials and Methods

Tissue Samples

Frozen tissue samples from 80 CRC patients were obtained from Shaoxing People's Hospital (Zhejiang, China) and Zhejiang Tumor Hospital (Zhejiang, China) in the study. The patients were diagnosed with CRC by pathologists' histological diagnosis and no one had ever received radiation or chemotherapy before surgery. Hematoxylin and eosin (H&E) stained slides were used to determine representative areas of invasive tumor. We examined each sample by microscope and there are over 80% of cancer cells presenting in each sample. The study was approved by ethical committees at Shaoxing People's Hospital and Zhejiang Cancer Hospital. Written informed consent forms were obtained from all participating individuals.

DNA Isolation, Bisulfite Modification and Quantitative Methylation Specific PCR (qMSP)

The details of DNA isolation, bisulfite modification and qMSP were as described previously [6]. E.Z.N.A.™ Tissue Kit (Omega Bio-Tek, Norcross, GA) was used to isolate genomic DNA according to the manufacturer's instructions. DNA concentration was determined by using Nanodrop2000 spectrophotometer (Thermal Scientific Co. Ltd., Wilmington, USA). Genomic DNA was chemically modified by bisulfite modification, which converted unmethylated cytosine to thymine, whereas methylated cytosine would not be change. SYBR green-based quantitative methylation specific PCR (qMSP) was performed by LightCycler® 480 (Roche Diagnostics, Mannheim, Germany) after DNA extraction and bisulfite conversion, aiming to quantify the extent of gene methylation. The primer sequences were shown in Table 1. The whole system of qMSP was add up to 10 μl containing 0.25 μl primers, 1 μl transformed DNA, 4 μl of ddH2O and 5 μl of 2 × SYBR Green Master Mix. PCR was conducted under the following conditions: 1 cycle at 95 °C for 10 min, 45 cycles at 95 °C for 20 s, specific annealing temperature (Table 1) for 20 s, 72 °C for 30 s, 1 cycle for the melting curve analysis at 95 °C of 15 s, 60 °C of 1 min, then increasing temperature at 0.11 °C per second to 95 °C, and a final extension stage at 40 °C for 10 min. *ACTB* was chosen as the internal reference, and methylated sperm DNA from a healthy individual by excess SssI methyltransferase (Thermo Fisher Scientiific, Uppsala, Sweden) was used as a positive control. The percentage of methylated reference (PMR) of the 2 genes in each sample was calculated by $2^{-\Delta\Delta Ct}$ quantification approach, in which $\Delta\Delta Ct$ = sample DNA ($Ct_{\text{target gene}} - Ct_{ACTB}$) − fully methylated DNA ($Ct_{\text{target gene}} - Ct_{ACTB}$) [21].

Luciferase Reporter Gene Assay

The human embryonic kidney 293 T (HEK293T) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin Solution. The target fragments of *SFRP1* and *SFRP2* were purified by Cycle Pure Kit (Omega, Norcross, GA, USA). The amplified genes and the plasmid were digested by XhoI and KpnI (New England Biolabs, Ipswich, MA). The pGL3 control vector (Promega, Madison city, WI, USA) containing SV40 promoter upstream of the luciferase gene was used as a positive control and the empty pGL3-Basic vector was used as a negative control. The target fragments were cloned to pGL3 promoter vector in the presence of DNA Ligation Kit (TaKaRa, Japan). Cells were prepared in 96-well plates and details of plasmid transfection were described in previous articles [22]. Luciferase activity was determined by the dual luciferase reporter gene assay system (Dual-Luciferase® Reporter Assay Systems, Promega, Madison city, WI, USA). Each test was carried out in triplicate.

Table 1 The primer sequences and amplification conditions in the qMSP experiments

Gene	Forward primer	Reverse primer	Product length (bp)	Annealing temperature (°C)
ACTB-1	GTGATGGAGGAGGT TTAGTAAGTT	CCAATAAAACCTAC TCCTCCCTTAA	129	56
ACTB-2	TGGTGATGGAGGAG GTTTAGTAAGT	AACCAATAAAACCT ACTCCTCCCTTAA	133	58
SFRP1	GAAGAGCGAGTAGA GGAA	ACACGAAACCATAA CGAAA	103	58
SFRP2	AAGAGCGAGTATAG GAAT	CCTACCAACCTACA ACTA	167	56

Data Mining Study

We extracted the 450 K array data of 395 CRC patients from the TCGA database. The methylation level of Methyl450 CpG site (cg15839448) was used to present the methylation of *SFRP1*. The mean methylation levels of cg05874561 and cg05774801 were used to present the methylation of *SFRP2*. And *SFRP1* and *SFRP2* methylation levels also were compared between 395 CRC tumor tissues and 48 non-tumor tissues. Moreover, we measured the association between *SFRP1* and *SFRP2* methylation and expression among 214 CRC tumor tissues according to the TCGA database. The expression data were retrieved from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo, accession no. GSE32323). We compared the expression changes of *SFRP1* and *SFRP2* in three CRC cell lines (COLO20, HCT116 and HT29) with and without 5′-AZA-deoxycytidine treatment.

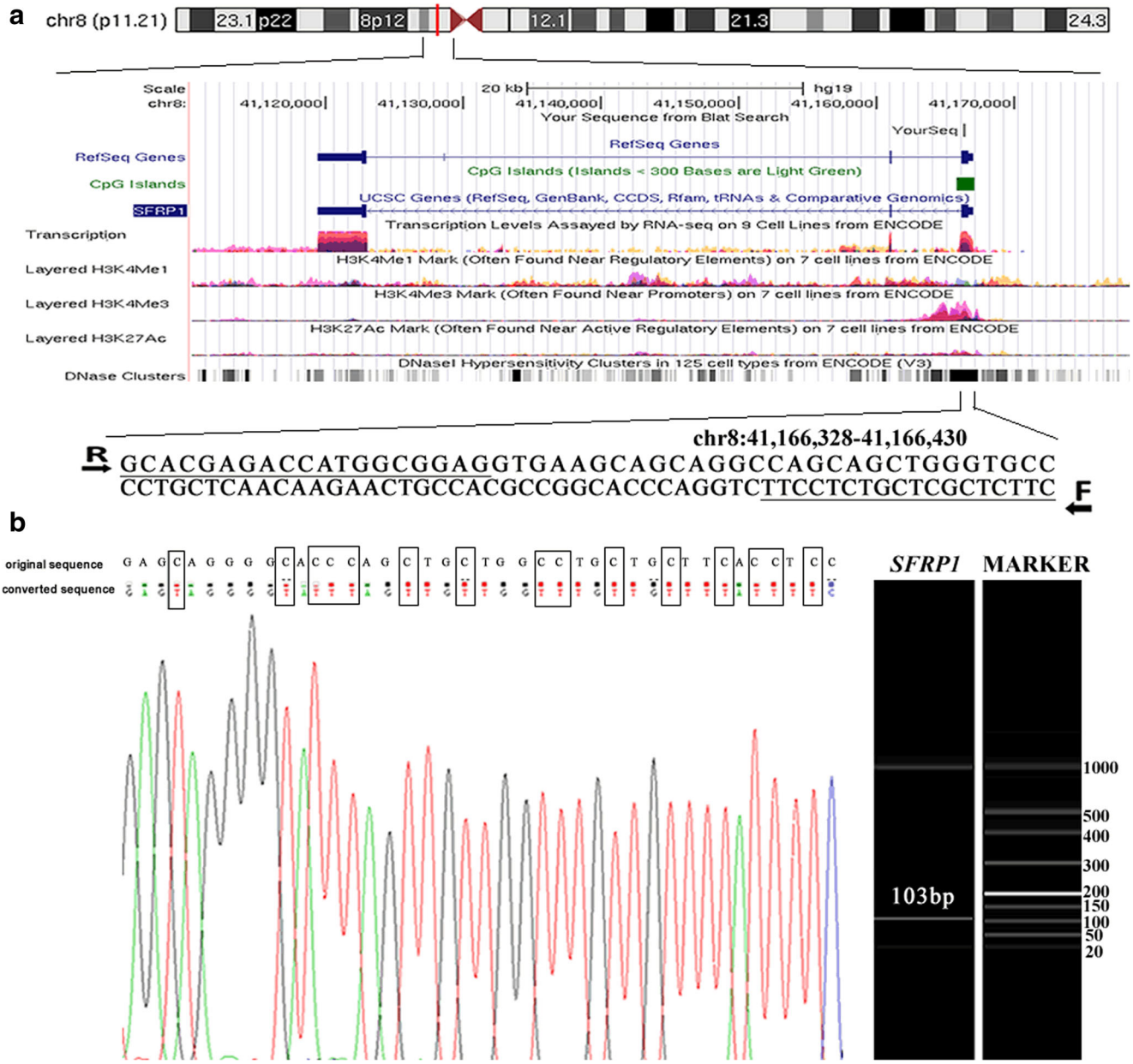

Fig. 1 Target sequences on *SFRP1* CpG island (CGI) region. **a** The genomic position and functional annotation of amplified fragment *SFRP1* from UCSC genome browser according to human 2009 (GRCh37/hg19) assembly. The qMSP primers were underlined. F: forward primer; R: reverse primer. **b** The top row of the sequence represented the original sequence, and the second row showed the converted sequence. And the framed base indicated that the cytosines were replaced by thymines (C to T conversion) in bisulfite-treated DNA. The picture on the right was the electrophoresis result of a representative qMSP product

Statistical Analysis

Spearman correlation test was used to determine the relationship between association between clinicopathological characteristics and gene methylation. Either independent sample T test or nonparametric test was used to detect the methylation differences between tumor tissues and non-tumor tissues. The diagnostic value of *SFRP1* and *SFRP2* methylation for CRC was evaluated by the receiver operating characteristics (ROC) test.

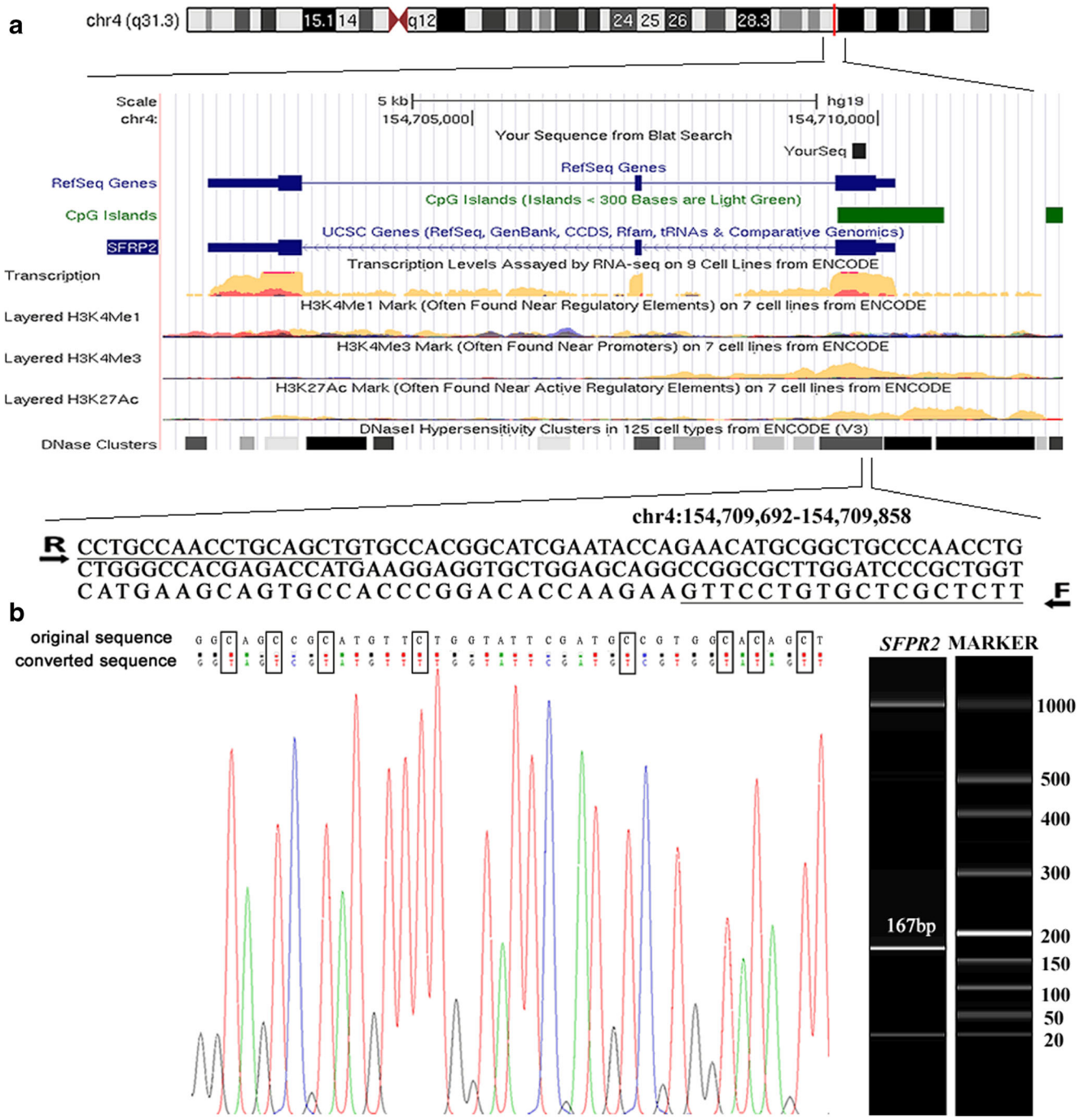

Fig. 2 Target sequences on *SFRP2* CpG island (CGI) region. **a** The genomic position and functional annotation of amplified fragment *SFRP2* from UCSC genome browser according to human 2009 (GRCh37/hg19) assembly. The qMSP primers were underlined. F: forward primer; R: reverse primer. **b** The top row of the sequence represented the original sequence, and the second row showed the converted sequence. And the framed base indicated that the cytosines were replaced by thymines (C to T conversion) in bisulfite-treated DNA. The picture on the right was the electrophoresis result of a representative qMSP product

$P < 0.05$ was considered statistically significant difference.

Results

We collected 80 pairs of CRC tumor samples and adjacent non-tumor samples for the detection of *SFRP1* and *SFRP2* methylation. The target fragments of *SFRP1* and *SFRP2* in the current methylation assay included 103 bp (hg19, chr8: 41166328–41,166,430, Fig. 1a) and 167 bp (hg19, chr4: 154709692–154,709,858, Fig. 2a), respectively. Meanwhile, we used the Sanger sequencing method to confirm that the amplified fragments matched the target sequences (Fig. 1b, Fig. 2b).

The methylation levels of *SFRP1* and *SFRP2* in tumor tissues were significantly higher than those in paired para-tumor tissues [PMR of *SFRP1*: 12.035% (1.710%, 50.498%) versus 2.225% (0.323%, 10.210%), $P = 2E\text{-}5$; PMR of *SFRP2*: 15.635% (0.675%, 103.675%) versus 5.905% (0.500%, 36.525%), $P = 0.014$; Fig. 3a]. Then, *SFRP1* and *SFRP2* hypermethylation was found in 56 out of 80 CRC patients and 45 out of 80 CRC patients, respectively. Moreover, we examined the correlation between the methylation of *SFRP1* and *SFRP2* and the clinicopathological features of CRC patients. However, no statistically significant correlation was found between the methylation of two genes with age, gender, differentiation, lymph node metastasis, tumor stage, tumor size, and tumor localization (Table 2).

In addition, we assessed the association between the clinicopathological features of CRC patients and tumor localization. In the present study, we did not find any statistically significant correlation of tumor localization with age, gender, differentiation, lymph node metastasis, or tumor stage ($P > 0.05$). Interestingly, there was a significant correlation between tumor localization and tumor size ($P = 0.001$). Tumors with size larger than 6 cm were more frequently in the colon cancer (17/39) than rectum cancer (5/41). More studies should be performed to explain the explicit relationship between the clinical characteristics of CRC and tumor localization.

Subsequently, we used ROC curve to calculate the diagnostic value of *SFRP1* and *SFRP2* methylation in CRC, respectively. The area under the curve of *SFRP1* is 0.669 (95% CI: 0.586–0.753), with a sensitivity of 60.0% and a specificity of 70.0%. And the area under the curve of *SFRP2* is 0.555 (95% CI: 0.465–0.645) with a sensitivity of 38.8% and a specificity of 77.5%.

According to our previous study, no significant promoter activity could be found for the recombinant plasmids of *SFRP1* and *SFRP2* fragments [6]. In the current study,

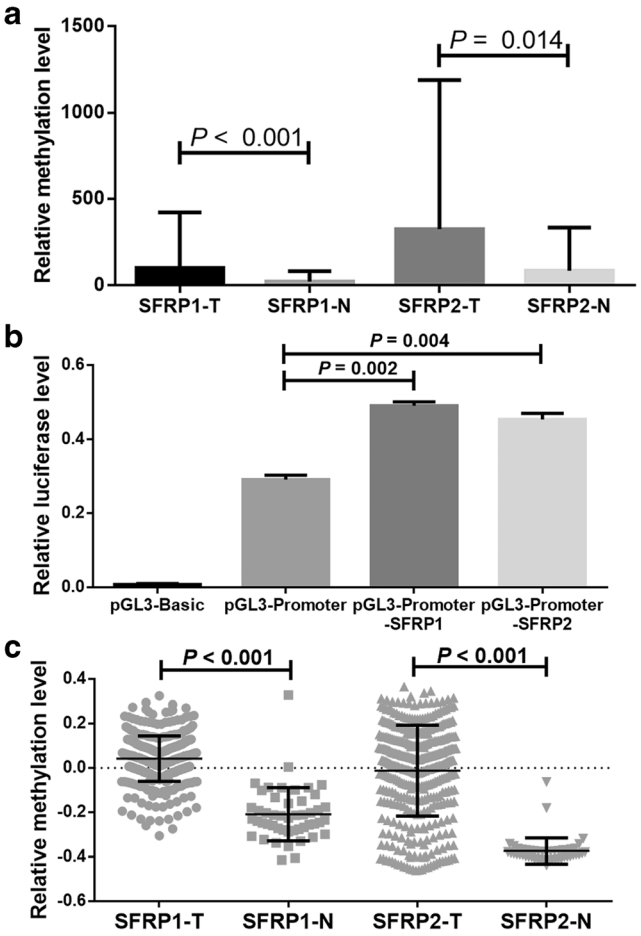

Fig. 3 The methylation levels of *SFRP1* and *SFRP2* in colorectal cancer. **a** Comparisons of *SFRP1* and *SFRP2* methylation levels between tumor tissues and paired adjacent non-tumor tissues in CRC patients. **b** Dual-luciferase reporter assay in HEK-293 T cell line. **c** Comparisons of *SFRP1* and *SFRP2* methylation levels between tumor tissues and adjacent non-tumor tissues in CRC patients from TCGA database. T stands for tumor tissues; N stands for adjacent non-tumor tissues. Statistical values and the bar were presented as median with interquartile range

luciferase reporter gene assay showed that the recombinant plasmids with *SFRP1* and *SFRP2* fragments could enhance the promoter activity (*SFRP1*: $P = 0.002$, *SFRP2*: $P = 0.004$, Fig. 3b).

The data from Cancer Genome Atlas (TCGA) was downloaded to validate the result. Our data mining study showed that the relative methylation levels of *SFRP1* (cg 05874561) were significantly higher in CRC tissues than that in paired adjacent non-tumor tissues [the mean β value: 0.0467 (−0.0184, −0.1038) versus −0.2278 (−0.2789, −0.1522), $P = 7E\text{-}21$, Fig. 3c]. And *SFRP1* hypermethylation yielded an AUC of 0.947 (95% CI: 0.904–0.990) with a sensitivity of 88.4% and a specificity of 95.8%. Similarly, *SFRP2* methylation in tumor tissues was significantly higher than that in paired adjacent tissues [the mean β value:

Table 2 Association of SFRP1 and SFRP2 methylation with clinical characteristics in colorectal cancer patients

Clinical characteristics	Variable	Number	SFRP1		SFRP2	
			PMR	*P* value	PMR	*P* value
Age	≤60	42	6.445 (1.553,22.605)	0.065	15.635(0.685,66.723)	0.992
	>60	38	28.490(1.928,111.175)		16.610(0.383,229.075)	
Gender	Male	51	13.070(1.680,73.860)	0.688	13.420(0.670,100.300)	0.729
	Female	29	10.110(1.805,37.285)		21.350(0.530,195.900)	
Differentiation	Poorly differentiated	15	12.660(0.840,38.310)	0.802[a]	51.980(3.610,104.800)	0.183[a]
	Moderately + Well differentiated	63	10.800(1.950,51.170)		13.420(0.360,151.900)	
Disease stage	I + II	41	8.370(1.445,54.580)	0.567	13.420(0.680,76.700)	0.958
	III + IV	39	13.820(2.590,45.610)		17.850(0.390,239.900)	
Tumor Size	≤6 cm	58	11.010(1.653,46.328)	0.532	12.750(0.383,116.575)	0.623
	>6 cm	22	14.305(1.893,137.100)		28.695(0.723,125.400)	
Lymph nodes metastasis	Positive	37	5.420(1.445,37.285)	0.077	4.620(0.375,48.905)	0.296
	Negative	43	17.460(3.860,109.000)		21.140(0.750,214.200)	
Tumor Localization	Rectum	41	11.220(1.625,36.485)	0.237	11.550(0.680,53.790)	0.397
	Colon	39	13.070(1.800,117.700)		26.615(0.635,232.825)	

PMR stands for the percentage of methylated reference, and data is presented as median (interquartile range). *P* value is calculated by Spearman test

[a] The information of two cases' differentiation is lost

0.0267 (−0.1424, 0.1407) versus −0.3831 (−0.4006, −0.3693), $P = 5E\text{-}24$, Fig. 3c]. And *SFRP2* hypermethylation yielded an AUC of 0.914 (95% CI: 0.886–0.942) with a sensitivity of 87.8% and a specificity of 95.8%.

In addition, *SFRP1* and *SFRP2* methylation was negatively correlated with the mRNA expression displayed by TCGA data mining (*SFRP1*: $r = -0.432$, $P = 3.682E\text{-}11$; *SFRP2*: $r = -0.478$, $P = 1E\text{-}13$, Fig. 4a). Using the data of GEO database (GSE32323), we found that *SFRP1* and *SFRP2* expression in three CRC cell lines (COLO20, HCT116 and HT29) was increased after 5′-AZA-deoxycytidine treatment (Fig. 4b). All the evidence indicated that *SFRP1* and *SFRP2* methylation might regulate gene expression.

Discussion

Despite of the improvement of technology, early diagnosis of CRC is still hindered for the lack of effective method. As the most common tumor marker, carcinoembryonic antigen (CEA) and carbohydrate antigen 19–9 (CA19–9) showed 33% sensitivity and 11% sensitivity at early stages of CRC patients, respectively [23, 24]. In addition, the positive rate of fecal occult blood test (FOBT) in normal control group and CRC was 9.1% and 33.3%, respectively [25]. In this study, the methylation levels of *SFRP1* and *SFRP2* in CRC tissues were found to be significantly higher than those in the adjacent non-tumor tissues. *SFRP1* and *SFRP2* hypermethylation was found in 56 out of 80 (70%) and 45 out of 80 (56%) CRC patients, respectively. Our findings provided candidate biomarkers for the diagnosis of CRC.

Altered DNA methylation patterns were considered as a screening method to diagnose cancer and predict tumor progression and prognosis which could be discovered by specific test methods in advance [26]. There are some DNA hypermethylation genes, such as *SLC5A8*, *ITGA4*, *SFRP2*, *CDKN2A*, *HLTF*, and *MGMT*, seeming to play a crucial role in colon carcinogenesis [27]. *SFPR1* and *SFRP2* were reported to be useful in early detection or CRC [28]. In the current study, *SFRP1* methylation yielded an AUC of 0.669 (sensitivity: 60.0%; specificity: 70.0%) and *SFRP2* methylation yielded an AUC of 0.555 (sensitivity: 38.8%; specificity: 77.5%) in CRC. Compared with CEA, CA19–9 and FOBT, *SFRP1* and *SFRP2* hypermethylation might sever as more sensitive biomarkers for CRC scanning. CpG island methylator phenotype (CIMP) status seems to be an emerging biomarker, which detected multiple genes methylation in CRC [29]. Therefore, we could combine *SFRP1* and *SFRP2* hypermethylation with other specific biomarkers to create a new CIMP-based diagnostic panel.

Multiple levels of regulation were existed in the Wnt signaling pathway. The secreted frizzled-related protein (SFRP) family is one of the antagonists of two functional families controlling the activation of Wnt signaling [30]. *SFRP1*, encoding a tumor suppressor protein, acts as a modulator of the Wnt signaling pathway [16]. *SFRP1* could prevent the Wnt proteins from combining with the Fz receptors, and thus stop the initiation of

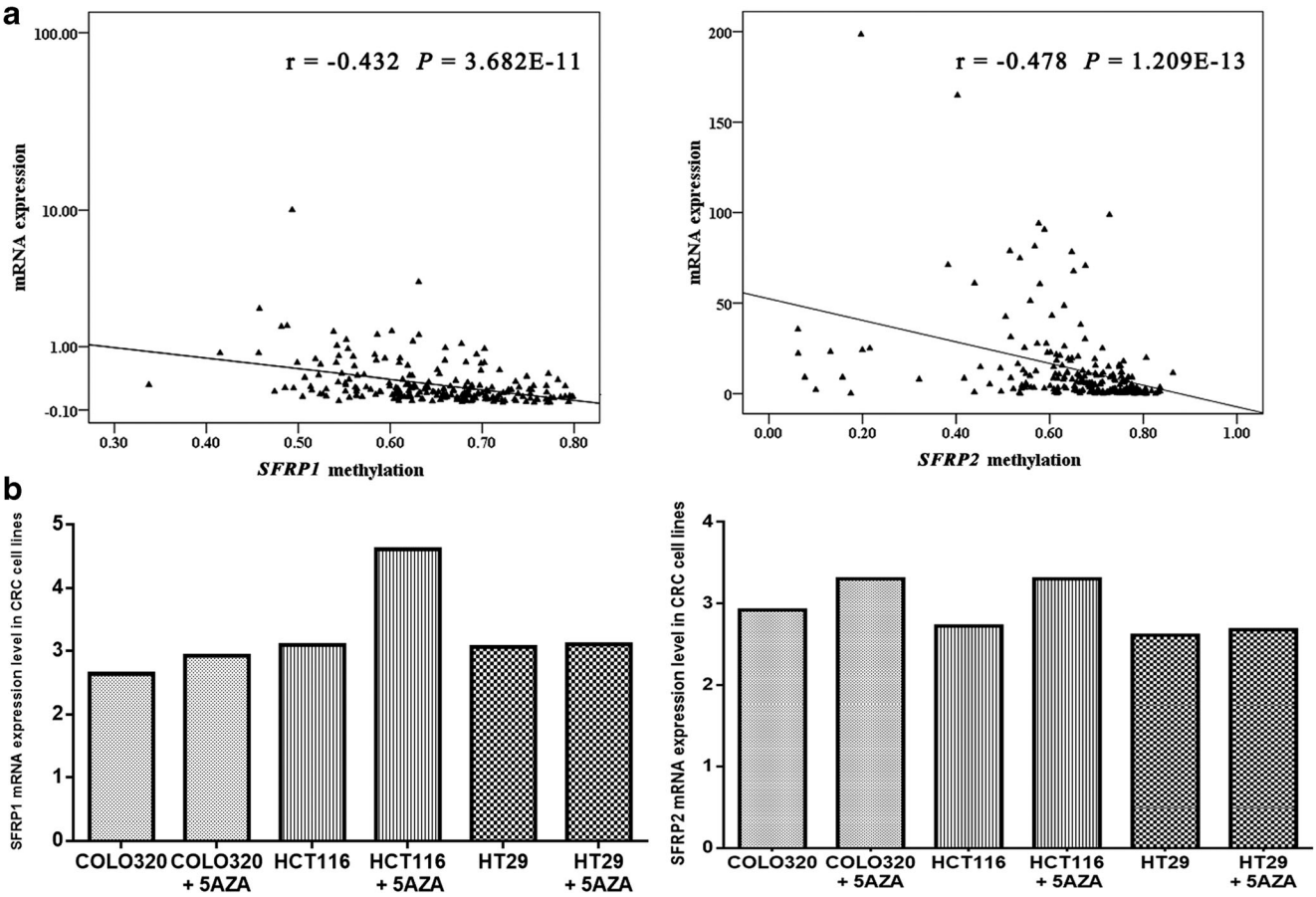

Fig. 4 Correlation between methylation and gene expression for *SFRP1* and *SFRP2* in CRC. **a** Correlation between methylation and gene expression for *SFRP1* and *SFRP2* among 214 CRC individuals from TCGA dataset. **b** The comparisons of *SFRP1* and *SFRP2* mRNA expression levels in CRC cell lines (COLO320, HCT116 and HT29) with and without 5′-AZA-deoxycytidine treatment from GEO database (accession number GSE32323)

signaling cascade [31]. The suppressed *SFRP1* could remarkably down-regulate p-β-catenin, and it will affect the proliferation and invasion in CRC cells [32]. Similarly, *SFRP2* was related to Wnt pathway and it was shown to accelerate cancer cell invasion and growth in tumor progression [33]. In the current study, analyses of TCGA and GEO databases showed that *SFRP1* and *SFRP2* hypermethylation were inversely correlated with gene expression. Therefore, we speculated that *SFRP1* and *SFRP2* hypermethylation might be involved in carcinogenesis through silencing the tumor-suppressor genes through Wnt pathway.

There are some limitations in our study. We only obtained tumor tissues in the current study. The blood-based strategies have the advantages of minimally-invasiveness compared to endoscopie. However, only one blood-based biomarker, the circulating cell-free DNA (cfDNA) methylation biomarker Epi proColon30, has been approved by FDA with a sensitivity of 72.2% with a specificity of 80.8% [34]. What's more, Harada et al. suggested that DNA methylation of *miR-124-3*, *LOC386758* and *SFRP1* in bowel lavage fluid (BLF) obtained during colonoscopy may be a promising biomarker for CRC (AUC = 0.834; sensitivity: 82%; specificity: 79%) [35]. Thus, the diagnostic value of *SFRP1* and *SFRP2* hypermethylation in blood and BLF samples should be assessed in the future. In addition, future studies should be performed to explore the potential regulatory mechanism between DNA methylation and gene expression.

In summary, *SFRP1* and *SFRP2* hypermethylation might be useful diagnostic biomarkers for the detection of CRC. Further studies on the detailed mechanisms of *SFRP1* and *SFRP2* hypermethylation should be explored to elaborate their role in the carcinogenesis of CRC.

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Author's Contribution SD and HH contribute to the conception, design and final approval of the submitted version. TW, CZ, RP and HH contribute to interpretation of data and completion of figures and Tables. YY, BL and JZ contribute to performing the experiments and analyzing the data. HH, TW and SD contribute to writing the paper. All the authors have read and approved the final manuscript.

Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflicts of interest.

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