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ABOUT COVER

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AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 642 experts in gastroenterology and hepatology from 59 countries.

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ORIGINAL ARTICLE

Basic Study

Involvement of methylation-associated silencing of formin 2 in colorectal carcinogenesis

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Abstract

AIM

To investigate whether promoter methylation is responsible for the silencing of formin 2 (*FMN2*) in colorectal cancer (CRC) and to analyze the association between *FMN2* methylation and CRC.

METHODS

We first identified the expression levels and methylation levels of *FMN2* in large-scale human CRC expression datasets, including GEO and TCGA, and analyzed the relationship between the expression and methylation levels. Then, the methylation levels in four CpG regions adjacent to the *FMN2* promoter were assessed by MethylTarget™ assays in CRC cells and in paired colorectal tumor samples and adjacent nontumor tissue samples. Furthermore, we inhibited DNA methylation in CRC cells with 5-Aza-2′-deoxycytidine and assessed the expression of *FMN2* by qRT-PCR. Last, the association between *FMN2* methylation patterns and clinical indicators was analyzed.

RESULTS

A statistically significant downregulation of *FMN2* expression in large-scale human CRC expression datasets was found. Subsequent analysis showed that a high frequency of hypermethylation occurred in the *FMN2* gene promoter in CRC tissues; operating characteristic curve analysis revealed that *FMN2* gene methylation had a good capability for discriminating between CRC and nontumor tissue samples (AUC = 0.8432, $P < 0.0001$). MethylTarget™ assays showed that CRC cells and tissues displayed higher methylation of these CpG regions than nontumor tissue samples. Correlation analysis showed a strong inverse correlation between methylation and *FMN2* expression, and the inhibition of DNA methylation with 5-Aza significantly increased endogenous *FMN2* expression. Analysis of the association between *FMN2* methylation patterns and clinical indicators showed that *FMN2* methylation was significantly associated with age, N stage, lymphovascular invasion, and pathologic tumor stage. Notably, the highest methylation of *FMN2* occurred in tissues from cases of early-stage CRC, including cases with no regional lymph node metastasis (N0), cases in stages Ⅰ and Ⅱ, and cases with no lymphovascular invasion, but the methylation level began to decrease with tumor progression. Additionally, *FMN2* promoter hypermethylation was more common in patients > 60 years old and in colon cancer tissue.

CONCLUSION

FMN2 promoter hypermethylation may be an important early event in CRC, most likely playing a critical role in cancer initiation, and can serve as an ideal diagnostic biomarker in elderly patients with early-stage colon cancer.

Key words: Formin 2; Colorectal cancer; Methylation; Methylation-associated silencing; Early-stage cancer

Core tip: Colorectal cancer (CRC) is the leading cause of cancer death in the world. We identified a statistically significant downregulation of formin 2 (*FMN2*) expression in large-scale human CRC expression datasets and our clinical samples. Then, we first showed that a high frequency of hypermethylation occurred in the *FMN2* gene promoter, which is responsible for the downregulation of *FMN2* expression. Additionally, the highest methylation of *FMN2* occurred in tissues from cases of early-stage CRC and patients > 60 years old. *FMN2* hypermethylation may be an important early event in CRC and can serve as an ideal diagnostic biomarker in elderly patients with early-stage CRC.

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INTRODUCTION

Colorectal cancer (CRC) is a common malignancy and a major cause of incidence and mortality in many countries, especially more developed countries[1]. Over the years, researchers have identified CRC as a gradual process that lasts for several years, since it begins as a single mutation in a cell until it becomes a detectable malignancy[2]. Therefore, the main secondary preventive strategy for CRC is the early detection of preneoplastic or neoplastic lesions in the large bowel[3].

Formin 2 (FMN2) is a member of the formin homology protein family; the encoded protein is thought to have essential roles in the organization of the actin cytoskeleton and in cell polarity[4]. More recently, *FMN2* was reported to be involved in cancer, for example, serving as a potential oncogene in leukemia[5]. FMN2 can enhance the expression of the cell cycle inhibitor p21 by preventing its degradation. In addition, *FMN2* is induced by the activation of other oncogenes, hypoxia, and DNA damage[6]. To date, only two studies have explored the relationship between FMN2 and CRC. The first study was in 2012; to identify the genetic determinants of colon tumorigenesis, Liu *et al*[7] and colleagues carried out a genome-wide association study of azoxymethane-induced colon tumorigenesis and subsequently confirmed through fine mapping that *FMN2* is associated with colon tumor susceptibility. Another study[8] assessed the expression of FMN2 in tumor and adjacent nontumor tissue by immunohistochemistry, with results showing that FMN2 was predominantly localized in the cytoplasm of tumor cells; that the rate of positive FMN2 protein expression in the CRC and paracarcinoma tissues was 53.73% (180/335) and 80.90% (271/335), respectively, with a significant difference ($P < 0.05$); and that the level of the FMN2 protein in CRC patients was associated with tumor differentiation, TNM stage, and lymph node metastasis. However, the mechanism leading to the downregulation of FMN2 expression in CRC has not been studied. In this article, we tried to show that the high frequency of hypermethylation occurring in the promoter of the *FMN2* gene in CRC tissues is responsible for the silencing of *FMN2,* and we further explored the association between *FMN2* methylation and clinical indicators. We found that the highest level of *FMN2* methylation occurred in early-stage CRC tissues; thus, FMN2 may provide a new biomarker for the secondary prevention of CRC.

MATERIALS AND METHODS

Cell lines and tissue samples

Human CRC cell lines (SW620 and SW480) were cultured in L15 medium (KeyGEN BioTECH, Nanjing, China) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel), and HCT116 and HT-29 cells were cultured in McCoy's 5A medium (KeyGEN BioTECH, Nanjing, China) supplemented with 10% FBS. Cells were grown in a 5% CO_2 cell culture incubator at 37 ℃.

Table 1 Clinical materials used in this article

No.	Sex	Age	Lymph nodes	TNM	Pathology	Differentiation	MSI	Organ
1	M	67	5 (14)	T4aN2aM0	Adenocarcinoma	Moderate	No	Rectum
2	M	53	0 (22)	T2N0M0	Adenocarcinoma	Moderate	-	Rectum
3	M	72	4 (20)	T4aN2aM0	Adenocarcinoma	Poor	-	Right colon
4	M	52	0 (16)	T3N0M0	Adenocarcinoma	Moderate	-	Rectum
5	M	52	3 (25)	T3N1bM0	Adenocarcinoma	Moderate	-	Sigmoid colon
6	F	57	0 (15)	T4aN0M0	Adenocarcinoma	Moderate	-	Left colon
7	F	51	0 (14)	T4aN0M0	Adenocarcinoma	Moderate	No	Rectum
8	F	66	19 (27)	T3N2bM0	Adenocarcinoma	Poor	No	Rectum
9	F	71	0 (16)	T4bN0M1b	Adenocarcinoma	Moderate	No	Rectum

Table 2 Details of the four CpG regions in the CpG islands of formin 2

Target	TSS	Start	End	Length	Target strand	Distance2TSS
FMN2_1	240255184	240255353	240255531	179	+	169
FMN2_2	240255184	240255809	240256007	199	+	625
FMN2_3	240255184	240256241	240256483	243	+	1057
FMN2_4	240255184	240256757	240257017	261	+	1573

Start: The starting position of the product on the reference genome; TSS: The mRNA transcription initiation site; End: The end position of the product on the reference genome; Length: the product length; Target strand: The product orientation; Distance2TSS: The distance from the product to the TSS.

Table 3 Primers used for the MethylTarget™ assays

Primer name	Primer
FMN2_1_F	GAGGGTYGGGATGGTTTGAG
FMN2_1_R	CCCCCRCTCCCCTTCTTT
FMN2_2F	GAGTGTGYGGATTTTTTTGAGGT
FMN2_2R	AAATATCTAAAAACAAATCCTCTTACTCC
FMN2_3F	GATTTGTTYGAGAGTTTGGTTGT
FMN2_3R	AAACRCATCCTCAAAAACATCCT
FMN2_4F	TTTTTGAGTYGAGGGTTTAGAATTG
FMN2_4R	CACRTTCTAAAAACCATCCRCAAC

Clinical samples were obtained from patients treated at the Third Xiangya Hospital of Central South University (Hunan, China) under informed consent and approval by the Ethics Committee of Central South University (more details about the clinical samples can be found in Table 1).

RNA purification and reverse transcription-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, United States). The reverse transcription reaction was performed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan), and this kit includes reagents for reverse transcription and for the removal of genomic DNA (DNase Ⅰ treatment). cDNA was amplified with KOD SYBR® qPCR mix (TOYOBO, Japan) on a LightCycler® 480 Ⅱ system (Roche) according to the manufacturer's instructions (*FMN2* primers: forward, GCGAACGCTGTTGGAGAAG and reverse, CTGATTACACGGTTCCCTGAAG).

Treatment with 5-aza-2'-deoxycytidine

Cells were grown in appropriate culture conditions. For demethylation treatment, colorectal cells were treated with 5-aza-2'-deoxycytidine (5-Aza) (Sigma, United States) for 96 h (5 μmol/L), with daily replacement of the drug and medium. Untreated cells were used as a control group.

DNA extraction and bisulfate treatment

DNA was isolated with an Easypure Genomic DNA kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The DNA concentration was assessed by spectrophotometry and confirmed by gel electrophoresis; DNA was stored at -20 ℃. An EZ DNA Methylation Gold™ Kit (Zymo Research Corporation, CA, United States) was used to convert all unmethylated cytosine to uracils.

DNA methylation analysis

MethylTarget™ assays (targeted bisulfite sequencing) developed by Genesky BioTech (Shanghai, China) were carried out as previously described[9,10]. Briefly, CpG islands adjacent to the promoter region of the *FMN2* gene were analyzed, and based on these CpG islands, four CpG regions from CpG islands in *FMN2* were sequenced (the details, including the relative distance from the transcriptional start site, amplification primers, and product size, of these CpG regions can be found in Tables 2 and 3). Genomic DNA was converted with bisulfite, and PCR was performed to amplify the targeted DNA sequences. The products were sequenced on an Illumina MiSeq benchtop sequencer (Illumina, CA, United States).

Bioinformatics

Cosmic[11], a database cataloguing somatic mutations in human cancer, was used to assess methylation mutations in human cancer tissues. Datasets for CRC, including GEO GSE20842, GSE8671, and GSE4183, as well as TCGA[12], were used to assess the expression level

of *FMN2*. Data about DNA methylation, the patients, and the samples were downloaded from the TCGA database (https://cancergenome.nih.gov/).

Statistical analysis

GraphPad Prism 7 software was used to analyze the data. The methylation and expression levels in normal, adenoma, and cancerous tissues, as well as the methylation level in different clinical materials, were assessed by two-tailed unpaired Student's *t*-tests; box-whisker plots depict the means, 1st and 3rd quartiles, and minimum/maximum. The error bars in the figures represent SDs. Spearman's correlation coefficient (*r*) was used to determine the correlation. Overall survival (OS) and disease-free survival (DFS) were estimated using the Kaplan-Meier method. Receiver operating characteristic curves were constructed based on the level of *FMN2* methylation. The data were considered nonsignificant for $P > 0.05$.

RESULTS

Analysis of FMN2 expression in large-scale human CRC expression datasets

Previous studies demonstrated that *FMN2* is underexpressed in CRC. To further confirm the expression characteristics of *FMN2* in CRC, we expanded our analysis to published, large-scale human CRC expression datasets. GSE20842, which contains 65 paired samples of tumor and mucosal tissue samples from 65 CRC patients, was used to assess *FMN2* expression; we found a statistically significant downregulation of *FMN2* expression from normal colorectal tissue to carcinoma tissue ($P < 0.0001$) (Figure 1A). An identical pattern was observed in human CRC samples from TCGA ($P < 0.0001$); however, it is noteworthy that we found that the expression level of *FMN2* was significantly lower in early-stage CRC tissues (stages Ⅰ + Ⅱ *vs* stages Ⅲ + Ⅳ, $P = 0.02$) (Figure 1B), which indicates that *FMN2* downregulation may be an important early event in CRC. To confirm this finding, GSE8671, which contains 32 colorectal adenoma and corresponding normal colonic mucosa samples, was downloaded to analyze *FMN2* expression; we found a statistically significant downregulation of *FMN2* expression from normal colorectal tissue to adenoma tissue ($P < 0.0001$) (Figure 1C). In addition, we downloaded the GSE4183 dataset, which contains colonic biopsies of 15 patients with CRC, 15 patients with adenoma, and 8 healthy normal controls, to simultaneously assess the reduction of *FMN2* in adenomas and cancer tissues. As shown in Figure 1D**,** adenoma tissues displayed a greater reduction in expression ($P = 0.0002$) than tumor tissues ($P = 0.01$).

High frequency of hypermethylation occurs in the FMN2 gene promoter in CRC tissues

Somatic mutation in cancer is an important reason for the aberrant expression of genes, so the Catalogue of Somatic Mutations in Cancer (COSMIC), the world's largest and most comprehensive resource for exploring the impact of somatic mutations in human cancer, was used to analyze the somatic mutations of *FMN2* in CRC tissues. The result indicated that methylation was the main somatic mutation and that *FMN2* gene promoter hypermethylation occurred in 37.37% of CRC tissues. In addition, COSMIC indicated that *FMN2* methylation mainly occurred in CRC tissues and was the most common somatic mutation among all human tumor tissues and that *FMN2* was one of the top 20 genes with an extremely high frequency of hypermethylation in CRC. Then, *FMN2* gene promoter DNA methylation profiles were downloaded from TCGA; the results revealed a statistically significant hypermethylation of *FMN2* in tumor tissues compared with adjacent nontumor tissue samples ($P < 0.0001$) (Figure 1E). We next assessed the accuracy of the *FMN2* methylation signature for the detection of CRC *via* receiver operating characteristic curve analysis, and the analysis revealed a good capability for discriminating between CRC and nonneoplastic tissue specimens (AUC = 0.8432, 95%CI: 0.8022-0.8841; $P < 0.0001$) (Figure 1F).

Analysis of the features of methylation-associated FMN2 silencing in CRC tissue and cells

The majority of research indicates that promoter hypermethylation is a key mediator underlying the downregulation of gene expression. The high frequency of hypermethylation in the *FMN2* gene promoter and the significant inverse correlation between methylation levels and the expression of *FMN2* in 372 CRC tissues (Figure 2A) remind us that methylation is the main cause of *FMN2* gene silencing. To investigate this topic, nine paired colorectal tumor samples and adjacent nontumor tissue samples (for details, see Table 1) were selected for RT-qPCR and MethylTarget™ assays. We first examined *FMN2* expression using RT-qPCR in these paired samples. A significant reduction in *FMN2* expression was observed in seven of the nine CRC tissues (Figure 2B). Then, based on the CpG islands adjacent to the *FMN2* promoter region, four CpG regions (*FMN2*-1, *FMN2*-2, *FMN2*-3, and *FMN2*-4, the details of which can be found in Table 2) from CpG islands were amplified and sequenced. The result demonstrated that CRC tissues revealed a stronger methylation pattern than noncancerous tissues and identified three CpG regions, namely, *FMN2*-2, *FMN2*-3, and *FMN2*-4, with a statistically significant difference ($P = 0.0069$, $P = 0.0094$, and $P = 0.0005$, respectively) (Figure 2C-F; Table 2). Notably, tumor tissues (cases 1, 3-7, and 9) exhibiting lower *FMN2* expression than the corresponding noncancerous tissues displayed an increase in methylation at these islands compared with the noncancerous tissues, whereas CRC cases 2 and 8, with moderate FMN2 expression, revealed a methylation pattern similar to that of the corresponding noncancerous tissues. Correlation analysis revealed a strong inverse correlation between the methylation of island 2 ($r = -0.86$), island 4 ($r = -0.71$), and island 3 ($r = -0.78$)

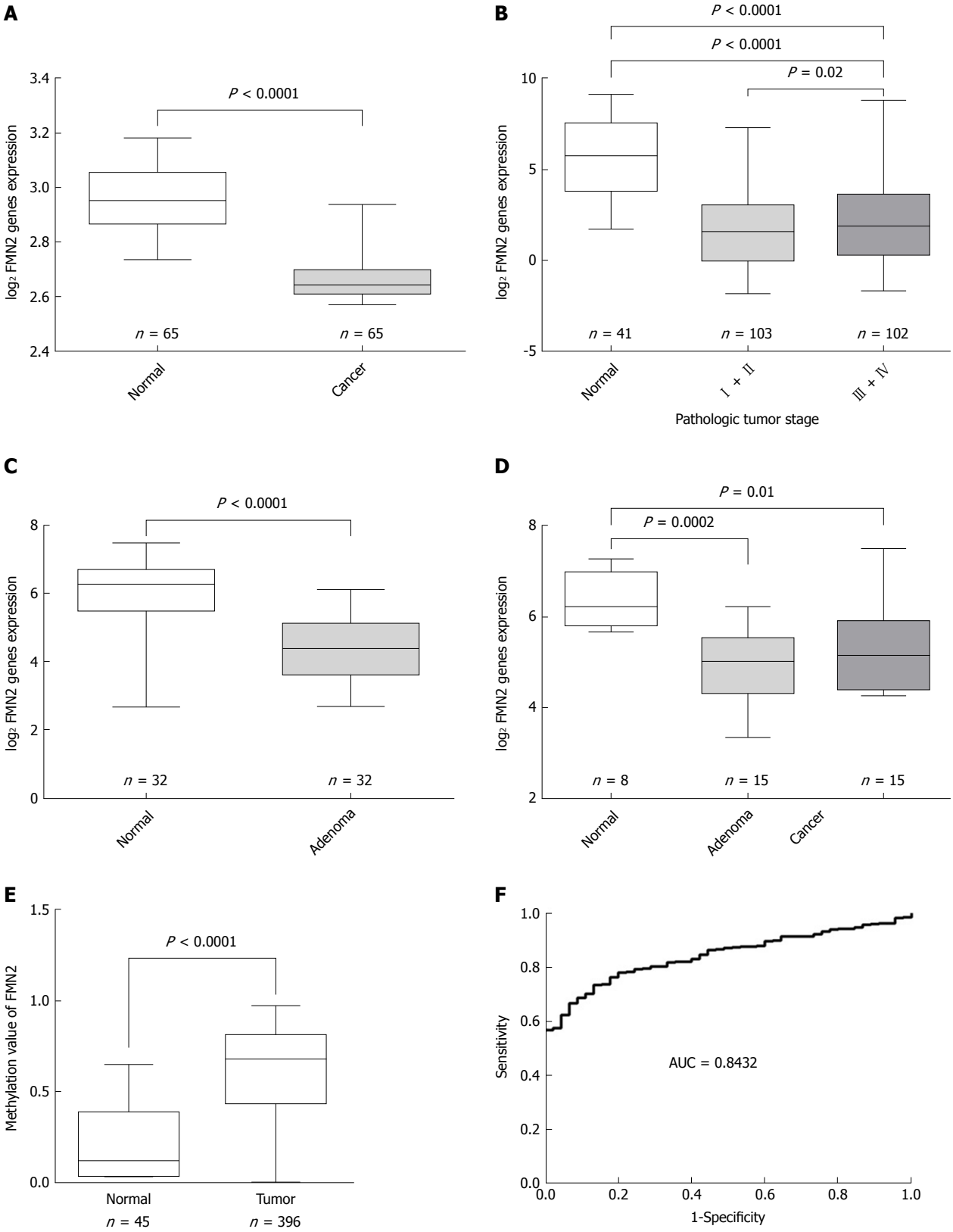

Figure 1 Colorectal tissues show decreased formin 2 expression and a high frequency of hypermethylation in the formin 2 promoter. A: Normalized expression of formin 2 (*FMN2*) mRNA in carcinoma and adjacent normal tissues, presented as box-whisker plots (unpaired *t*-test, GEO: GSE20842); B: Normalized expression of *FMN2* mRNA in tissue from different stages of carcinoma (according to the AJCC Cancer Staging Manual) and in normal tissue, presented as box-whisker plots (unpaired *t*-test, colorectal cancer samples from TCGA); C: Normalized expression of *FMN2* mRNA in adenoma and corresponding normal colonic mucosal tissues, presented as box-whisker plots (unpaired *t*-test, GEO: GSE8671); D: Normalized expression of *FMN2* mRNA in normal, adenoma, and carcinoma tissues, presented as box-whisker plots (unpaired *t*-test, GEO: GSE4183); E: The FMN2 gene shows an increased methylation level in colorectal cancer tissues compared with normal tissues (unpaired *t*-test, colorectal cancer samples from TCGA); F: Receiver operating characteristic curve analysis was used to assess the clinical diagnostic utility of *FMN2* DNA methylation for the prediction of colorectal cancer.

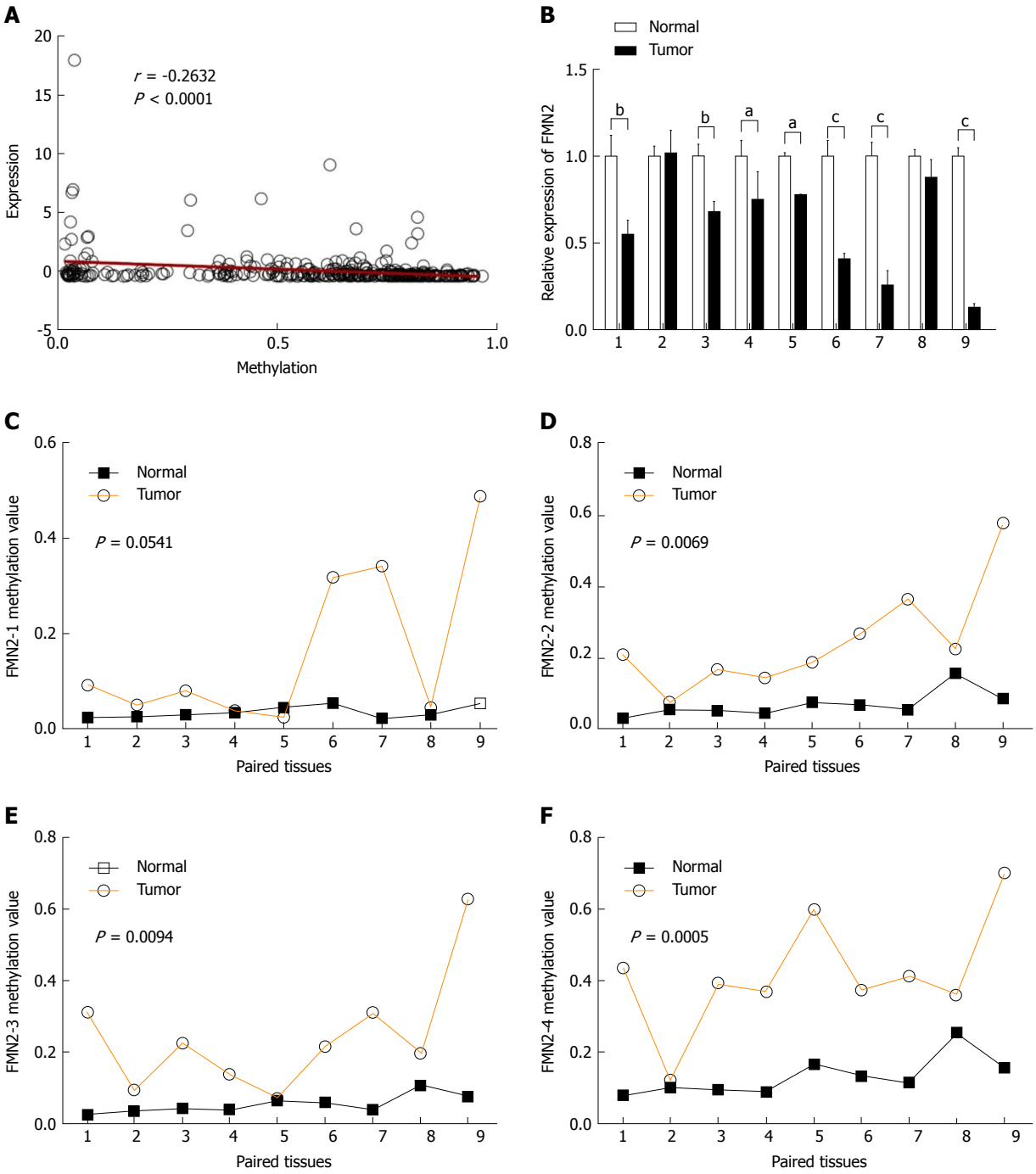

Figure 2 Hypermethylation of formin 2 correlates with decreased expression of formin 2 during colorectal cancer. A: The expression of the formin 2 (*FMN2*) gene is significantly inversely correlated with the methylation level (Pearson correlation analysis); B: Quantitative real-time RT-PCR for *FMN2* was carried out on nine colorectal cancer surgical specimens (filled bars) and paired noncancerous tissues (unfilled bars). The expression levels in the tumor samples were independently calculated relative to those in the nontumor samples, which are normalized to 1. Vertical bar, SD; $^{a}P < 0.05$, $^{b}P < 0.03$, $^{c}P < 0.01$ (unpaired *t*-test); C-F: Results of MethylTarget™ assays on nine paired noncancerous tissues and colorectal cancer tissues. Four CpG regions (*FMN2*-1, *FMN2*-2, *FMN2*-3, and *FMN2*-4) from CpG islands adjacent to the FMN2 promoter were sequenced (unpaired *t*-test. More details about the four CpG regions can be found in Table 2).

and *FMN2* expression (data not shown). In addition, we assessed the methylation level of each CG site in CpG island regions and found that 8 of 14 CG sites in *FMN2*-1 and all CG sites in *FMN2*-2 (13/13), *FMN2*-3 (23/23), and *FMN2*-4 (27/27) revealed a significantly stronger methylation pattern in the tumor tissues than in the corresponding noncancerous tissues (Table 4).

We next wondered whether promoter hypermethylation at these loci and *FMN2* expression are causally related. To answer this question, four CRC cell lines (HCT116, HT29, SW480, and SW620) were selected for MethylTarget™ assays. The result revealed a high level of DNA methylation in all CRC cell lines, whereas only limited methylation was observed in normal colon tissue from a healthy individual (Figure 3A). Then, we treated HCT116, HT29, and SW480 cells with 5-Aza;

Table 4 Methylation level of each CG site in the CpG island regions

Target	Position	Type	*P* value (*t*-test)	OR (L95-U95) (logistic)	C mean	N mean
FMN2_1	25	CG	0.007774578	2.1731 (0.9323-5.0653)	0.17	0.04
FMN2_1	27	CG	0.01061292	1.5266 (0.8980-2.5953)	0.16	0.03
FMN2_1	30	CG	0.1359111	1.3535 (0.7970-2.2986)	0.15	0.03
FMN2_1	32	CG	0.01875771	1.7416 (0.8662-3.5016)	0.16	0.04
FMN2_1	35	CG	0.03998355	1.2722 (0.8959-1.8068)	0.18	0.05
FMN2_1	38	CG	0.01419169	1.6993 (0.8462-3.4125)	0.16	0.03
FMN2_1	46	CG	0.003990128	2.2454 (0.9140-5.5161)	0.15	0.02
FMN2_1	90	CG	0.1134924	1.3103 (0.6401-2.6825)	0.16	0.03
FMN2_1	108	CG	0.06252571	1.2532 (0.7873-1.9948)	0.17	0.04
FMN2_1	114	CG	0.1134924	1.1858 (0.8641-1.6273)	0.18	0.05
FMN2_1	117	CG	0.05030852	1.3499 (0.8867-2.0550)	0.19	0.04
FMN2_1	123	CG	0.01061292	2.2247 (0.9162-5.4017)	0.16	0.03
FMN2_1	126	CG	0.09391197	1.2852 (0.7471-2.2110)	0.16	0.03
FMN2_1	134	CG	0.01875771	2.4645 (0.6195-9.8050)	0.16	0.02
FMN2_2	27	CG	0.000493624	1.3442 (1.0433-1.7321)	0.28	0.07
FMN2_2	51	CG	0.01419169	1.2762 (0.9670-1.6843)	0.17	0.04
FMN2_2	63	CG	0.007774578	1.4062 (0.9872-2.0030)	0.37	0.25
FMN2_2	70	CG	0.000781571	1.3753 (1.0378-1.8226)	0.24	0.05
FMN2_2	74	CG	0.000493624	1.3079 (1.0283-1.6636)	0.31	0.07
FMN2_2	78	CG	0.000287947	1.3676 (1.0603-1.7639)	0.25	0.05
FMN2_2	81	CG	0.00185109	1.3630 (0.9664-1.9224)	0.21	0.05
FMN2_2	107	CG	0.000493624	1.3025 (0.9942-1.7063)	0.24	0.06
FMN2_2	131	CG	0.007774578	1.2706 (0.9662-1.6708)	0.15	0.03
FMN2_2	137	CG	0.002756067	1.3002 (0.9986-1.6928)	0.21	0.05
FMN2_2	143	CG	0.00123406	1.3743 (1.0297-1.8341)	0.20	0.04
FMN2_2	164	CG	0.000287947	1.3527 (1.0108-1.8101)	0.29	0.07
FMN2_2	170	CG	0.00123406	1.2431 (1.0019-1.5424)	0.28	0.08
FMN2_3	24	CG	0.005635541	1.3854 (0.9994-1.9207)	0.20	0.05
FMN2_3	31	CG	0.005635541	1.4241 (0.9731-2.0841)	0.23	0.04
FMN2_3	47	CG	0.002756067	1.3776 (0.9695-1.9574)	0.24	0.06
FMN2_3	50	CG	0.003990128	1.3787 (1.0219-1.8599)	0.22	0.05
FMN2_3	57	CG	0.00123406	1.3592 (1.0212-1.8091)	0.25	0.06
FMN2_3	60	CG	0.003990128	1.5630 (0.9433-2.5897)	0.19	0.04
FMN2_3	68	CG	0.00185109	1.3474 (1.0273-1.7673)	0.23	0.06
FMN2_3	75	CG	0.00123406	1.3787 (0.9962-1.9082)	0.26	0.06
FMN2_3	80	CG	0.00123406	1.3582 (0.9518-1.9380)	0.24	0.05
FMN2_3	92	CG	0.00185109	1.3665 (0.9746-1.9159)	0.23	0.05
FMN2_3	98	CG	0.00123406	1.3200 (0.9414-1.8509)	0.27	0.06
FMN2_3	105	CG	0.000781571	1.4801 (0.9296-2.3566)	0.22	0.04
FMN2_3	107	CG	0.007774578	1.2402 (1.0141-1.5168)	0.21	0.05
FMN2_3	119	CG	0.00123406	1.4326 (0.9810-2.0921)	0.19	0.04
FMN2_3	131	CG	0.000493624	1.7790 (0.9589-3.3006)	0.22	0.04
FMN2_3	147	CG	0.007774578	1.3343 (0.9443-1.8856)	0.20	0.04
FMN2_3	153	CG	0.000493624	1.5405 (0.9677-2.4523)	0.22	0.05
FMN2_3	158	CG	8.23E-05	1.9417 (0.8981-4.1979)	0.31	0.08
FMN2_3	170	CG	4.11E-05	1714240.0000 (0.0000-Inf)	0.32	0.08
FMN2_3	174	CG	4.11E-05	11577516733.0000 (0.0000-Inf)	0.34	0.08
FMN2_3	183	CG	0.000287947	1.6836 (0.9224-3.0731)	0.28	0.06
FMN2_3	187	CG	0.000287947	1.4526 (0.9861-2.1399)	0.27	0.06
FMN2_3	203	CG	0.000781571	1.4980 (0.9815-2.2861)	0.25	0.05
FMN2_4	36	CG	0.003990128	1.1409 (1.0248-1.2702)	0.41	0.14
FMN2_4	42	CG	0.002756067	1.2068 (1.0353-1.4068)	0.38	0.12
FMN2_4	47	CG	0.000781571	1.2264 (1.0309-1.4589)	0.37	0.10
FMN2_4	61	CG	0.005386639	1.1552 (1.0206-1.3075)	0.38	0.10
FMN2_4	66	CG	0.0003108	243.9222 (0.0000-Inf)	0.40	0.09
FMN2_4	69	CG	8.23E-05	1.7294 (0.6520-4.5874)	0.42	0.09
FMN2_4	72	CG	0.003990128	1.2209 (1.0296-1.4477)	0.4	0.13
FMN2_4	80	CG	4.11E-05	2515571.0000 (0.0000-Inf)	0.48	0.18
FMN2_4	84	CG	0.00185109	1.2366 (1.0241-1.4931)	0.39	0.12
FMN2_4	94	CG	0.002756067	1.2121 (1.0363-1.4178)	0.38	0.12
FMN2_4	98	CG	0.003990128	1.1618 (1.0236-1.3186)	0.36	0.11
FMN2_4	110	CG	0.00185109	1.1712 (1.0253-1.3378)	0.44	0.19
FMN2_4	123	CG	0.000781571	1.2218 (1.0321-1.4463)	0.40	0.10
FMN2_4	127	CG	0.00185109	1.2220 (1.0324-1.4464)	0.39	0.11
FMN2_4	131	CG	8.23E-05	1.3179 (1.0259-1.6931)	0.38	0.08
FMN2_4	136	CG	0.003990128	1.1979 (1.0209-1.4054)	0.34	0.09
FMN2_4	150	CG	4.11E-05	1217702.0000 (0.0000-Inf)	0.54	0.22

FMN2_4	152	CG	0.003990128	1.1644 (1.0196-1.3297)	0.39	0.12
FMN2_4	166	CG	0.000164541	1.3446 (0.9896-1.8269)	0.45	0.16
FMN2_4	168	CG	0.001710676	1.2557 (1.0089-1.5628)	0.44	0.16
FMN2_4	176	CG	4.11E-05	26059.6300 (0.0000-Inf)	0.52	0.21
FMN2_4	186	CG	0.000781571	1.1735 (1.0224-1.3469)	0.54	0.2
FMN2_4	189	CG	0.003990128	1.1348 (1.0221-1.2600)	0.49	0.18
FMN2_4	213	CG	0.00185109	1.1572 (1.0241-1.3076)	0.47	0.14
FMN2_4	221	CG	0.002756067	1.1534 (1.0276-1.2947)	0.45	0.13
FMN2_4	234	CG	0.00185109	1.1981 (1.0181-1.4099)	0.32	0.08
FMN2_4	236	CG	0.000493624	1.2631 (1.0149-1.5720)	0.36	0.08

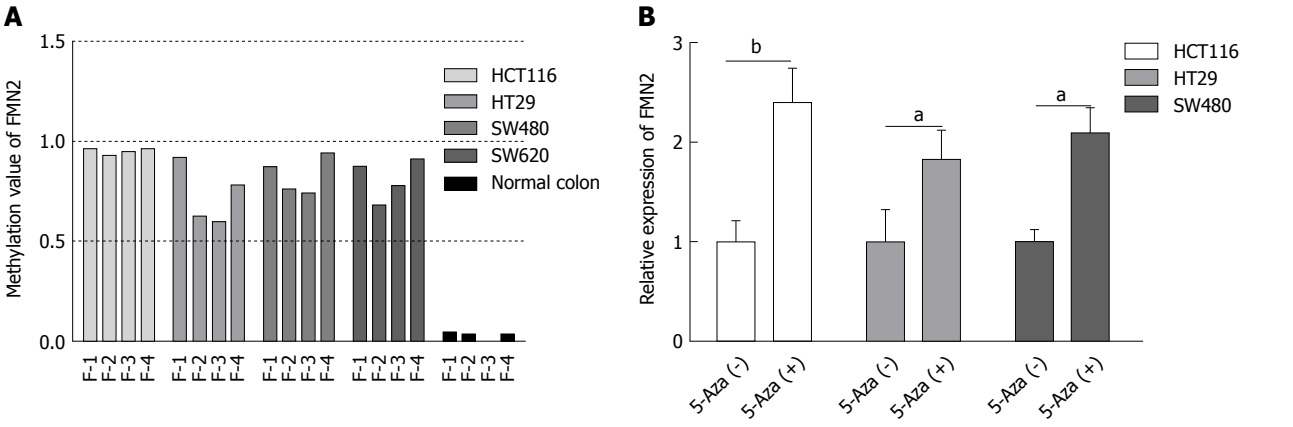

Figure 3 Promoter hypermethylation and formin 2 expression are causally related. A: Results of MethylTarget™ assays on four colorectal cancer cell lines and normal colon cells from a healthy individual; B: The inhibition of DNA methylation with 5-Aza significantly increased the endogenous *FMN2* expression (unpaired *t*-test).

the inhibition of DNA methylation by 5-Aza significantly increased the endogenous *FMN2* expression (Figure 3B).

FMN2 promoter hypermethylation is an early event in CRC

To analyze the clinicopathological significance of *FMN2* promoter methylation, we divided the patients into a high-methylation group and a low-methylation group according to the median value of methylation. The association between clinicopathological factors and *FMN2* methylation was analyzed (Table 5). High FMN2 methylation was associated with age (P = 0.0006), N stage (P = 0.0293), pathologic tumor stage (P = 0.0161), and lymphovascular invasion (P = 0.0180). Notably, the association analysis showed that the highest methylation level of *FMN2* occurred in tissues from cases of early-stage CRC, including cases with no regional lymph node metastasis (N0), cases in stages Ⅰ and Ⅱ, and cases with no lymphovascular invasion; the methylation level began to decrease with tumor progression. To understand our conclusions more intuitively, we then performed unpaired *t*-tests based on the pathological data. An identical pattern is shown in Figure 4A-C; DNA methylation of the *FMN2* gene increased from normal tissues to cancer tissues, reaching the highest level in early-stage cancer (N0, stages Ⅰ and Ⅱ, and no lymphovascular invasion) and decreasing stepwise in advanced-stage carcinomas. However, it is worth noting that the methylation level of *FMN2* in advanced-stage carcinomas is still significantly higher than that in normal tissue (P < 0.0001). Additionally, we analyzed the methylation level of *FMN2* according to the primary tumor site, and the result showed that there were statistically significant differences among the means (P < 0.0001) and that tumors located in the sigmoid colon and rectum had a relatively low *FMN2* methylation level (Figure 4D). Furthermore, we found that *FMN2* promoter hypermethylation was not associated with OS or DFS (Figure 4E and F). All these results indicated that *FMN2* promoter hypermethylation mainly occurred in elderly and early-stage patients with colon cancer.

DISCUSSION

In this article we first reported that a high frequency of hypermethylation occurred in the promoter of the *FMN2* gene in CRC tissues and that hypermethylation was responsible for the silencing of *FMN2*, which suggests that the epigenetic silencing of *FMN2* may be an important event in CRC. To the best of our knowledge, although *FMN2* was one of the top 20 genes with an extremely high frequency of hypermethylation in CRC (COSMIC, https://cancer.sanger.ac.uk/cosmic), this study is the first and only to investigate the regulation of the *FMN2* gene by methylation.

Previously, studies and investigations of publicly available datasets in FireBrowse (http://firebrowse.org/) have demonstrated differential *FMN2* RNA expression in human tumors, depending on the tumor type. For example, *FMN2* is overexpressed in approximately 95% of pre-B acute lymphoblastic leukemias[5] but is underexpressed in kidney renal clear cell carcinoma

Table 5 Relationship between formin 2 methylation levels and clinicopathological data

Factor	No.	*FMN2*		*P* value
		Low, *n* (%)	High, *n* (%)	
Age (yr)				
< 60	136	66 (45.83)	70 (28.69)	0.0006[1]
⩾ 60	252	78 (54.17)	174 (71.31)	
Gender				
Male	210	72 (50.00)	138 (56.56)	0.2105
Female	178	72 (50.00)	106 (43.44)	
Height (cm)				
< 170	138	52 (48.60)	86 (47.25)	0.8250
⩾ 170	151	55 (51.40)	96 (52.75)	
Weight (kg)				
< 80	156	56 (49.12)	100 (51.55)	0.6812
⩾ 80	152	58 (50.88)	94 (48.45)	
T				
T1	11	5 (3.47)	6 (2.47)	0.4830
T2	54	16 (11.11)	38 (15.71)	
T3	270	106 (73.61)	164 (67.77)	
T4	51	17 (11.81)	34 (14.05)	
N				
N0	212	69 (47.92)	143 (59.34)	0.0293[1]
N1 + N2	173	75 (52.08)	98 (40.66)	
M				
M0	264	98 (80.33)	166 (85.13)	0.2651
M1	53	24 (19.67)	29 (14.87)	
Stage				
Ⅰ + Ⅱ	197	62 (45.26)	135 (58.19)	0.0161[1]
Ⅲ + Ⅳ	172	75 (54.74)	97 (41.81)	
Lymphovascular invasion				
Yes	231	77 (60.63)	154 (72.99)	0.0180[1]
No	107	50 (39.37)	57 (27.01)	
Vascular invasion				
Yes	78	31 (24.80)	47 (22.71)	0.6627
No	254	94 (75.20)	160 (77.29)	
Perineural invasion				
Yes	59	20 (24.39)	39 (26.53)	0.7225
No	170	62 (75.61)	108 (73.47)	
Tumor status				
Tumor-free	246	86 (71.07)	160 (77.29)	0.2094
Tumor-present	82	35 (28.93)	47 (22.71)	
KRAS mutation				
Yes	28	8 (42.11)	20 (54.05)	0.3972
No	28	11 (57.89)	17 (45.95)	

[1]Indicates a statistically significant result. TNM stage was defined according to the AJCC Cancer Staging Manual. Tumor status: The state or condition of an individual's neoplasm at a particular point in time.

(FireBrowse); the mechanism underlying these phenomena has not been studied. In our research, we identified that *FMN2* is underexpressed in CRC tissues and were the first to explore the underlying mechanism. Epigenetic modifications of DNA, such as DNA promoter hypermethylation, have critical roles in mediating gene expression in mammalian development and human disease[13]. Methylation-mediated silencing of some genes in CRC has been reported in previous studies; for example, *Lgr5* methylation, by effecting *Lgr5* expression and CRC stem cell differentiation, may serve as a novel prognostic marker in CRC patients[14]. *SMYD3* promoter hypomethylation suppressed *SMYD3* expression and was associated with the risk of CRC[15]. Based on the fact that a high frequency of hypermethylation occurred in the *FMN2* gene promoter in CRC tissues, we found that both the downregulation of *FMN2* expression and the high frequency of hypermethylation occurred at the earliest stages of carcinogenesis; furthermore, these parameters had a significant inverse correlation in both our study and in published, large-scale human CRC electronic datasets. We also carried out MethylTarget™ assays in paired CRC tissues and inhibited DNA methylation with 5-Aza in CRC cells, which identified that hypermethylation and *FMN2* expression are causally related.

It is particularly important to determine which CpG islands adjacent to the promoter affect the expression of a gene, because this knowledge can provide directions for future research and potential targets for treatment. For example, Tavazoie SF[16] found that the *Mest/miR-335* promoter contained three CpG islands upstream of the transcriptional start site; among these CpG islands, island 3 demonstrated a strong inverse correlation between methylation and miR-335 expression

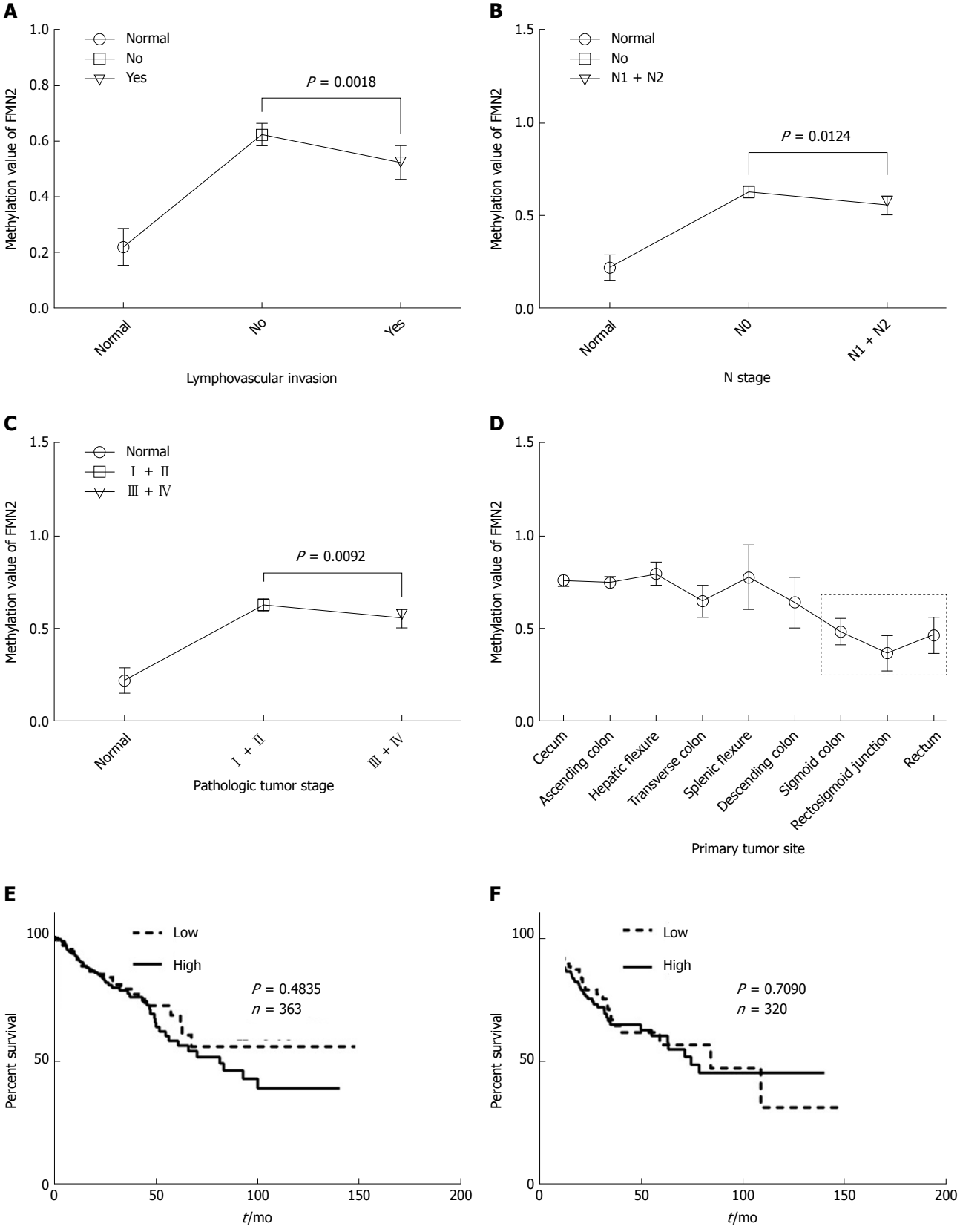

Figure 4 Formin 2 promoter hypermethylation mainly occurs in early-stage colon cancer patients. A: Formin 2 (*FMN2*) promoter methylation level in colorectal cancer (CRC) tissues with (yes) and without (no) lymphovascular invasion, presented as column means with 95%CI, mean connected. (unpaired *t*-test; normal: *n* = 45, CRC tissues with and without lymphovascular invasion: *n* = 231 and *n* = 107, respectively); B: *FMN2* promoter methylation level in CRC tissues with (N1 + N2) and without (N0) regional lymph node metastasis, presented as column means with 95%CI, mean connected (unpaired *t*-test, Normal: *n* = 45, CRC tissues with and without regional lymph node metastasis: *n* = 212 and *n* = 173, according to the AJCC Cancer Staging Manual); C: FMN2 promoter methylation level in CRC tissues from different stages, presented as column means with 95%CI, mean connected (unpaired *t*-test; Normal: *n* = 45, Stages Ⅰ + Ⅱ: *n* = 197, Stages Ⅲ + Ⅳ: *n* = 172, according to the AJCC Cancer Staging Manual); D: *FMN2* promoter methylation level in different organ sites, presented as column means with 95%CI, mean connected; E and F: *FMN2* hypermethylation has no effect on the overall survival or disease-free survival of CRC patients.

(r^2 = -0.81). The methylation status of island 3 plays a key role in inhibiting miR-335 expression. An identical pattern was observed in our study; we found that although the methylation status of *FMN2*-2, -3 and -4 was significantly increased in the eight CRC tissues, the methylation of *FMN2*-1 was not significantly higher than that in the paired nontumor tissues. All these findings suggest that during CRC progression, *FMN2* expression may be mainly mediated by the methylation of a specific CpG island region in the *FMN2* promoter.

In addition, we found that the hypermethylation of *FMN2* was significantly associated with age, N stage, pathologic tumor stage, and lymphovascular invasion. Notably, the association analysis showed that the highest methylation level of *FMN2* occurred in tissues from cases of early-stage CRC, including cases with no regional lymph node metastasis (N0), cases in stages Ⅰ and Ⅱ, and cases with no lymphovascular invasion and that the methylation level began to decrease with tumor progression. Identical results have been reported in previous research. In endometrial tumorigenesis, Schneider-Stock[17] found that DNA methylation of the *APC* gene increased from atypical hyperplasia (23.5%) to endometrial carcinoma, reaching its highest level of 77.4% in early-stage cancer (FIGO stages Ⅰ and Ⅱ) and decreasing stepwise to 24.2% in advanced-stage carcinomas (FIGO stages Ⅲ and Ⅳ). Epigenetic inactivation of the candidate tumor suppressor *USP44* is a frequent and early event in colorectal neoplasia[18], and *P16* methylation is an early event in cervical carcinogenesis[19]. All these studies, along with ours, have identified that DNA hypermethylation can be an early event in tumorigenesis. Hypermethylation most likely plays a critical role in cancer initiation, and creates an environment conducive to the overwhelming accumulation of simultaneous genetic and epigenetic mutations[20]. Additionally, we identified that patients younger than 60 years and patients with tumors located in the sigmoid colon and rectum exhibit relatively low *FMN2* methylation levels, which indicates that the methylation-associated silencing of *FMN2* may be involved in specific patients. In addition, Grady[21] identified that the patterns of DNA methylation in the colon vary by anatomical location, patient gender, and patient age.

In summary, we identified that the RNA expression of *FMN2* is reduced in CRC tissues and were the first to reveal that the hypermethylation of specific CpG islands adjacent to the *FMN2* promoter is the mechanism underlying *FMN2* silencing. Although the *FMN2* methylation is significantly stronger in tumor tissues than in paired nontumor tissues, the methylation level begins to decrease with tumor progression, which suggests that DNA hypermethylation is an early event in CRC tumorigenesis and can serve as a biomarker for the detection of CRC.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is a critical contributor to cancer mortality and morbidity, and some critical genes and pathways were important in the initiation and progression of CRC. Recent studies have shown that formin 2 (*FMN2*) may be down-regulated in CRC. Whether *FMN2* is abnormally expressed in CRC and what causes its abnormal expression are unclear. Revealing the role of FMN2 in CRC may provide potential therapeutic targets.

Research motivation

Epigenetic modifications of DNA, especially promoter hypermethylation, have critical roles in mediating some gene expression in the initiation and progression of CRC. Whether abnormal DNA methylation changes can occur in the promoter of the *FMN2* gene in CRC and whether such changes can be responsible for the silencing of *FMN2* are poorly known. So the main topic in this article is to try to solve this question that epigenetic silencing of *FMN2* may be an important event in CRC. All can provide the basis and direction for future research.

Research objectives

This study focuses on whether FMN2 is underexpressed in CRC, and whether methylation changes occur in CpG islands located in the promoter region of *FMN2*. If changes occur, what are the characteristics of fragments and CpG sites in these CpG islands? What is the core fragment of the change? Does methylation change silence the expression of *FMN2*, and is there a correlation between methylation changes in *FMN2* and clinical indicators of CRC? The answers to these questions can better explain the role of FMN2 in CRC.

Research methods

Large-scale human CRC expression datasets, including GEO and TCGA, were used to assess the expression levels and methylation levels of *FMN2* in CRC. Then, the mRNA levels of *FMN2* in our own clinical samples was analyzed by real-time quantitative polymerase chain reaction, and the methylation levels in four CpG regions adjacent to the *FMN2* promoter were assessed by MethylTarget™ assays in CRC cells and in paired colorectal tumor samples and adjacent nontumor tissue samples. Furthermore, we performed demethylation treatment in CRC cells with 5-Aza-2'-deoxycytidine and assessed the expression of *FMN2* by qRT-PCR. Last, the association between *FMN2* methylation patterns and clinical indicators was analyzed.

Research results

FMN2 is underexpressed in CRC, and the most obvious low expression occurs in early colon cancer tissues. Subsequent analysis coming from large-scale human CRC datasets showed that *FMN2* was one of the top 20 genes with an extremely high frequency of hypermethylation in CRC, methylation was the main somatic mutation, and *FMN2* gene promoter hypermethylation occurred in 37.37% of CRC tissues. Our own experiments confirmed that CRC cells and tissues displayed higher methylation levels in CpG regions of *FMN2* than nontumor tissue samples. Correlation analysis showed a strong inverse correlation between methylation and *FMN2* expression. Treatment of CRC cells with demethylation reagent, 5-Aza-2'-deoxycytidine, can significantly increase endogenous *FMN2* expression. The highest methylation of *FMN2* occurred in tissues from cases of early-stage CRC, including cases with no regional lymph node metastasis (N0), cases in stages I and II, and cases with no lymphovascular invasion. Additionally, *FMN2* promoter hypermethylation was more common in patients > 60 years and in colon cancer tissue.

Research conclusions

In this article we confirmed the low expression of *FMN2* in CRC and first reported that a high frequency of hypermethylation occurred in the promoter of the *FMN2* gene in CRC tissues and that hypermethylation was responsible for the silencing of *FMN2*. It is worth noting that we found that the low expression and hypermethylation of *FMN2* occurred more prominently in early colon cancer tissues, which suggests that DNA hypermethylation leading to the low expression of the *FMN2* gene may be an important early event in CRC, most likely playing a critical role in cancer initiation, and can serve as an ideal diagnostic biomarker in elderly patients with early-stage colon cancer.

Research perspectives

In the era of big data, the rational use of tumor databases such as TCGA, GEO, and COSMIC can provide us with valuable information. The emergence of some new technologies can provide more accurate data for our experiments.

For example, MethylTarget™ assays developed by Genesky BioTech used in this article can be used very well for methylation analysis. Involvement of the methylation-associated silencing of *FMN2* in colorectal carcinogenesis may be a valuable research direction. Future research can select more clinical specimens to explore whether it can be used as a good clinical diagnostic index, and can also develop demethylation treatment. The article on the relationship between *FMN2* and CRC is rare, so further study investigating whether FMN2 participates in the process of colorectal carcinogenesis and its underlying mechanisms are very important.

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