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Downregulation of fibulin-3 gene by promoter methylation in colorectal cancer predicts adverse prognosis

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Fibulin-3 gene has been identified as an antagonist of angiogenesis. We investigated the protein expression and promoter methylation status of fibulin-3 gene in colorectal cancer (CRC) and analyzed its correlation with clinicopathological factors. The study population enrolled 85 paired CRC specimens and adjacent normal tissues, as well as 32 cases of colorectal adenoma. Genomic DNA was extracted from paraffin-embedded samples using manual microdissection. Methylation-specific polymerase chain reaction (MSP) was used to determine the promoter methylation status and fibulin-3 gene expression was detected by immunohistochemistry. The results showed that, downregulation or silence of fibulin-3 protein was found in 57.6% (49/85) of CRC tissues, which was significantly higher than that of adjacent normal tissues (28.2%, 24/85) and colorectal adenoma (34.4%, 11/32) ($P<0.05$). Furthermore, 33 out of 85 (38.8%) CRC specimens showed hypermethylation in fibulin-3 promoter region, and fibulin-3 methylation was closely correlated with its loss of expression. Also, downregulation of fibulin-3 was associated with advanced stage ($P=0.008$) and lymph node metastasis ($P=0.013$). Survival analyses and Cox proportional hazard models indicated that fibulin-3 downregulation was an independent factor related to adverse overall survival (OS) and disease-free survival (DFS) of CRC. In conclusion, we found aberrant methylation caused fibulin-3 downregulation in CRC, and fibulin-3 downregulation was correlated with tumor stage, lymph node metastasis and poor survival, which maybe use as a potential prognostic factor for CRC.

Key words: *fibulin-3; methylation; immunohistochemistry; CRC; prognosis*

Colorectal cancer (CRC) is one of the most common cancers worldwide with approximately one million new cases occurring per year [1]. In the last two decades, the incidence of CRC has been increasing in China but the prognosis remains poor. It is clearly imperative to clarify the pathogenesis of CRC. Previous studies have shown that the formation and development of CRC is polygenic with multi-stage changes, in which oncogene mutation and tumor suppressor gene inactivation play important roles [2].

Fibulin-3 gene, also known as EFEMP1 (Epidermal growth factor-containing fibulin-like extracellular matrix protein 1), is a member of the fibulins gene family that consists of seven extracellular matrix (ECM) proteins [3]. Fibulins are widely distributed and localized at basal membranes, stroma and ECM fibers mediating cell to cell and cell to matrix communication [4]. While diverse functions of the fibulins gene family members have been reported in details [5-8], the role of fibulin-3 during development of tumors remains largely unknown. Albig *et al* [9] reported that fibulin-3 antagonized tumor angiogenesis and decreased the blood vessel growth and density in the tumor. Sadr-Nabavi *et al* [10] reported that the level of fibulin-3 expression decreased in sporadic breast cancers due to aberrant promoter methylation and correlated to poor survival. Similar results were found in lung cancer [11,12] and hepatocellular carcinoma [13]. However, other reports demonstrated that fibulin-3 overexpression induced the production of VEGF and promoted tumor growth and invasion in pancreatic adenocarcinoma [14] and malignant glioma cells [15], furthermore, fibulin-3 expression was found to be significantly associated with lymph node metastasis, vascular invasion and poor survival in cervical carcinoma [16]. These paradoxical effects of fibulin-3 in cancer prompted us to study clinical implications of fibulin-3 in CRC that has never been investigated.

Thus, in the current study, we determined the expression level and methylation status of fibulin-3 in 85 pairs of CRC and corresponding normal tissues, its clinicopathological significance was further evaluated.

Materials and methods

Study population. A total of 85 paired CRC specimens and their adjacent normal tissues, 32 colorectal adenoma specimens were enclosed in this study. All samples were formalin-fixed, paraffin-embedded, and diagnosed at the Department of Pathology, Yijishan Hospital and Yangzhou No.1 People's Hospital, between 2002 and 2005. Only patients with primary colorectal adenocarcinoma without neoadjuvant radiochemotherapy were included. The cancerous patients consisted of 52 males and 33 females, with a median age of 59 years, ranging from 42 to 83 years. Histologic diagnosis was established on standard H&E-stained sections according to the 2000 WHO classification system for tumors of digestive system, and tumor stage was determined according to the 2002 TNM staging guidelines suggested by the American Joint Committee on Cancer and the Union Internationale Contre le Cancer. Clinical follow-up data were available for all CRC patients, and follow-up periods for survivors ranged from 3 to 60 months, with a median follow-up time of 44 months. Ethical approval was obtained from the hospital and fully informed consent from all patients prior to sample collection.

Immunohistochemical staining. Formalin-fixed, paraffin-embedded samples used for immunohistochemistry were sectioned at 2 μm thickness. All the sections were deparaffinized using xylene, dehydrated by gradient ethanol, and then rehydrated with deionized water. Heatmediated antigen retrieval was run by autoclave treatment (120°C for 2 min in 1 mmol/l EDTA, pH 8.0) and then followed by cooling at room temperature. Incubation with the mouse anti-human-fibulin3 antibody (diluted 1:200, Santa Cruz, CA, USA) was performed overnight at 4°C. After washing with phosphate-buffered saline (PBS), the sections were then incubated with secondary antibody (Dako, Ely, UK) for 30 min at room temperature. Staining was performed with 3, 3-diaminobenzidine (DAB). Nuclei were counterstained with hematoxylin. PBS was used as a negative control for the staining reactions. The immunostaining results were evaluated independently by three pathologists. The percentage of positive cells was rated as follows: 0 score for 0–5%, 1 score for 6–25%, 2 scores for 26–50%, and 3 scores for more than 50%. The staining intensity was rated as follows: 0 score for no staining, 1 score for weak staining, 2 scores for moderate staining, and 3 scores for strong staining [17]. The scores from the percentage and intensity were added to an overall score, and the expression of the fibulin-3 protein in colorectal carcinomas with an overall score of 0-2 was designated as 'low', and with an overall score of 3-6 was designated as 'high'.

Bisulphite treatment of DNA, methylation-specific polymerase chain reaction (MSP). Genomic DNA from formalin-fixed pathology specimens was extracted using manual microdissection followed by the classic phenol/chloroform protocol [18]. After spectrophotometric quantitation, 1 μg of genomic DNA was bisulphite-treated with EZ-DNA methylation Gold Kit (Zymo Research, Orange, CA, USA), and finaly resuspended in 20 μl of TE buffer. Then polymerase chain reaction (PCR) was performed in a 25 μl volume containing 5 μl of DNA template, 10×Buffer, 0.15 mM dNTP, 0.1 mM each primer and 0.5U of Ex Taq Hot Start Version (Takara, Shiga, Japan). Primers of methylated and unmethylated fibulin-3 were listed in Table 1 [11]. PCR products were identified in 2% agarose gel and stained with ethidium bromide. Lymphocyte DNA, original or methylated *in vitro* by excessive CpG (SssI) methylase (New England Biolabs, Beverly, MA, USA), was used as unmethylation and methylation positive control. Water blank was used as a negative control. To verify the MSP results, stochastic bands from each target were gel-purified and cloned into pMD 18-T Vector (Takara) followed by automatic DNA sequencing provided by GeneScript (Nanjing, China).

Statistical analysis. The results were expressed as mean±s.d. of percentage where appropriate. The differential expressions of fibulin-3 between cancer and non-cancer specimens were calculated with Student's t-test. Differences in frequency were assessed by Chi-square test or Fisher's exact test. Overall survival curves were calculated using the Kaplan-Meier method and compared by log-rank testing. Multivariate Cox proportional hazard models were used to define the potential prognostic significance of individual parameter. Statistical tests were carried out using SPSS version 12.0 for windows (SPSS Inc., Chicago, IL, USA). $P<0.05$ was taken as statistical significance.

Results

Downregulation of fibulin-3 expression in CRC specimens. Firstly, immunohistochemistry assay was performed to detect the expression of fibulin-3 protein in the specimens section. In both normal colorectal mucosa and colorectal carcinoma, fibulin-3 expression was observed as dot-like staining in the cytoplasm, whereas it was not detected in the cellular nucleus. The representative immunohistochemical results are shown in Figure 1(A-D). In total, 36 tumors (42.4%) were clas-

Table 1. List of MSP primer sequences

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Product Size	PCR Condition
fibulin-3 (M)	GTAGTTTTAGGGGATCGTCGC	TCCCCGACACGCTACCTTCG	160 bp	58°C (40 cycles)
fibulin-3 (U)	GAGTAGTTTTAGGGGATTGTTGT	TCCCCAACACACTACCTTCA	162 bp	60°C (40 cycles)

M, Methylated; U, Unmethylated;

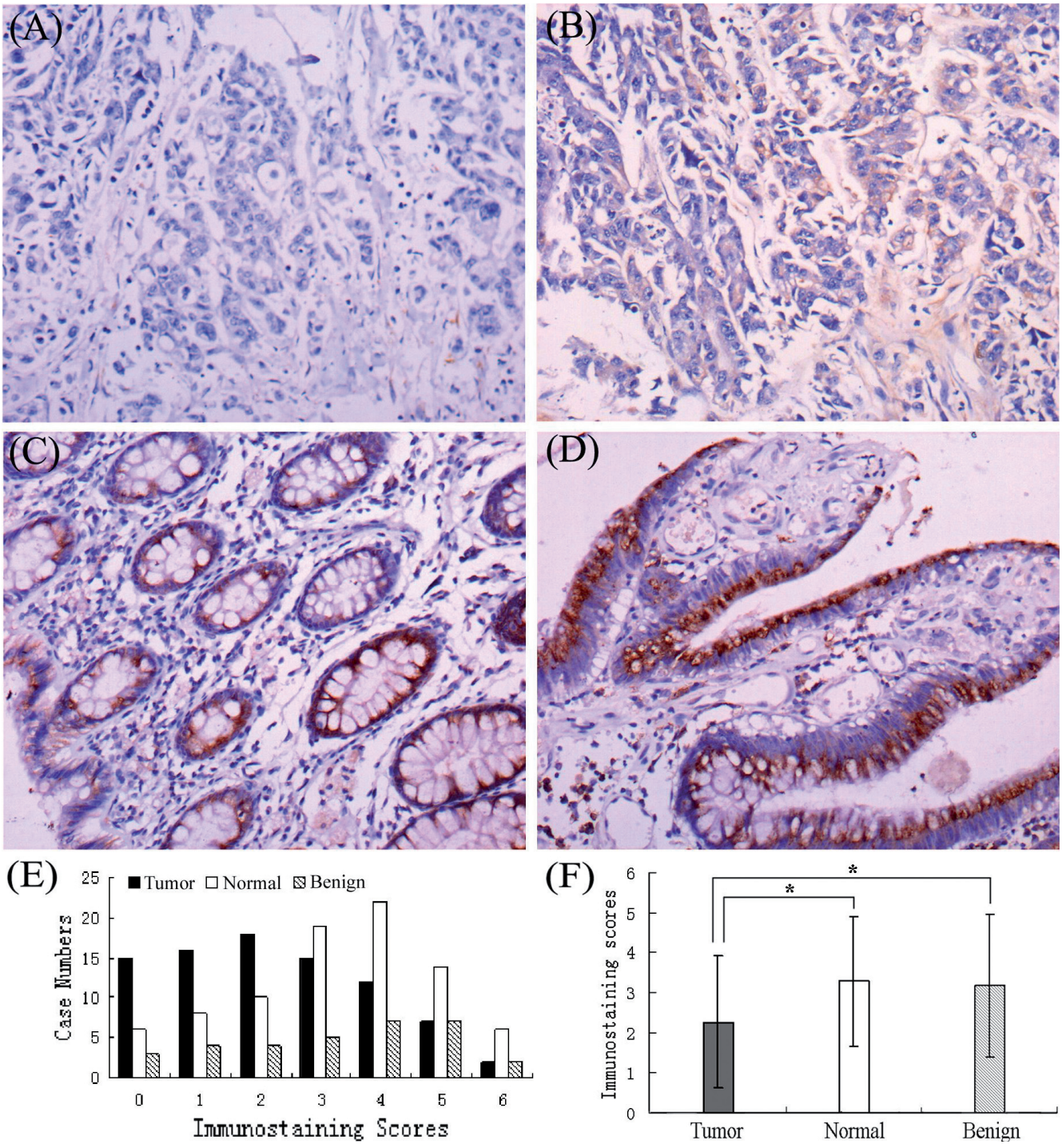

Figure 1. Protein expression levels of fibulin-3 in colorectal adenocarcinoma determined by immunohistochemical staining (EnVision, magnification ×200). Fibulin-3 protein was mostly silenced (A) or downregulated (B) in tumor tissues, while widely expressed in adjacent normal colorectal tissues (C) and colorectal adenoma (D). (E) Distribution of immunostaining scores per sample in tumor tissues, adjacent normal tissues and benign adenomas. (F) The difference of fibulin-3 expression between cancerous and non-cancerous tissues was significant (*P<0.001).

sified into the high fibulin-3 expression group, the remaining 49 tumors (57.6%), with constant low or negative fibulin-3 immunoreactivity were classified as the downregulation group.

The distribution of immunostaining scores per sample in tumor tissues, adjacent normal tissues and colorectal adenoma was shown in **Figure 1E**. The rate of fibulin-3 downregulation

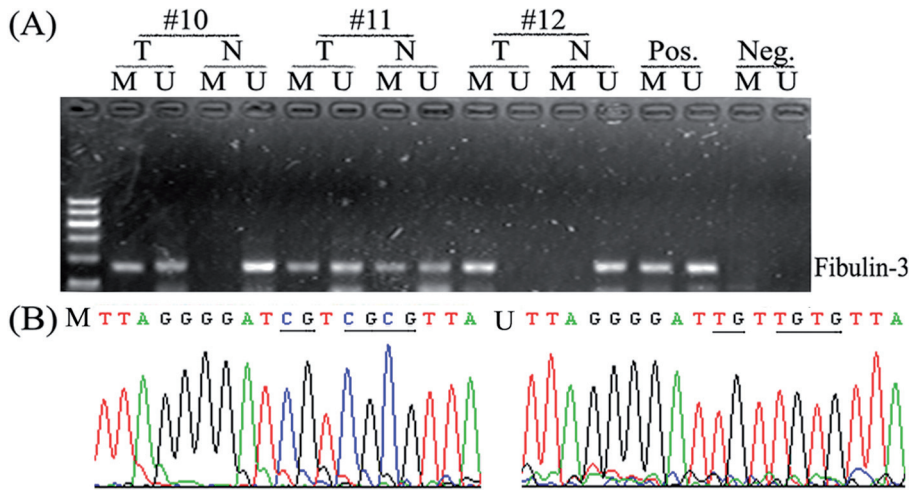

Figure 2. Methylation status of fibulin-3 gene in CRC tissues. (A) Typical agarose gel electrophoresis of MSP results in tissue samples. T, tumor tissues; N, adjacent normal lung tissues (patients 10-12). Fibulin-3 showed an aberrant methylation in tumor tissues of #10-12 and normal tissue of #11. (B) The MSP product of fibulin-3 was directly sequenced and confirmed. Methylated cytosines (C) would not be converted to uracil (T) and remained as C. (Genebank accession: AC010895.8).

in CRC, matched normal tissues and adenoma was 57.6%, 28.2% (24/85) and 34.4% (11/32), respectively, which showed significant difference between cancerous and non-cancerous tissues (P<0.05). In addition, the average immunostaining scores were significantly lower in tumor tissues (2.26 ± 1.65) than that in adjacent normal tissues (3.28 ± 1.62) and colorectal adenoma (3.18 ± 1.79) (Figure 1F, P<0.001).

Table 2. Relationship between clinicopathological featuress and fibulin-3 protein expression in 85 CRC patients

Patients	Cases	fibulin-3 expression		P-value
		Low	High	
Gender				0.362
Male	52	32	20	
Female	33	17	16	
Age				0.684
<55	24	13	11	
≥55	61	36	25	
Tumor size (cm)				0.237
<5	48	25	23	
≥5	37	24	13	
Tumor differentiation				0.181
Well	19	8	11	
Moderate	37	21	16	
Poor	29	20	9	
Tumor site				0.638
Proximal	22	14	8	
Distal	33	17	16	
Rectum	30	18	12	
Stage				0.008*
I/II	40	17	23	
III/IV	45	32	13	
Lymph metastasis				0.013*
N_0	41	18	23	
N_1/N_2	44	31	13	

*P<0.05

Methylation frequency of fibulin-3 gene in CRC specimens. To investigate the role of promoter methylation in downregulation of fibulin-3 in CRC, the methylation status of fibulin-3 gene was analyzed by MSP. We found that 33 out of 85 (38.8%) CRC specimens had hypermethylation in the promoter region of fibulin-3 gene, which was significantly higher than that of adjacent normal tissues (11.8%, 10/85; P<0.001) and adenoma (15.6%, 5/32; P=0.017). The representative MSP results are shown in Figure 2. Moreover, of the 33 methylated CRC tissue samples, 26 cases showed silence or downregulation of fibulin-3 expression, in contrast, 29 of the 52 unmethylated CRC specimens showed upregulation or no difference of fibulin-3 expression *vs* matched normal colorectal tissue. Thus, the downregulation of fibulin-3 protein expression in CRC was significantly correlated with its promoter methylation status (P=0.002). Notably, we also found that 7 patients with DNA methylation and normal fibulin-3 expression, but their methylation states were incomplete methylation, such as patient #10, #11 showed in Figure 2A.

Clinicopathological correlation of fibulin-3 expression in CRC. Next, we analyzed the correlation between the fibulin-3 protein expression and clinicopathological features of CRC patients. As shown in Table 2, downregulation of fibulin-3 gene was preferentially observed in patients with advanced pathological stage (42.5%, 17 of 40 in I/II stage *vs* 71.1%, 32 of 45 in III/IV stage; P=0.008) or lymph node metastasis (43.9%, 18 of 41 in N_0 *vs* 70.5% 31 of 44 in N_1/N_2; P=0.013). However, there were no correlations of fibulin-3 expression and patients' gender, age, tumor size, site and cellular differentiation.

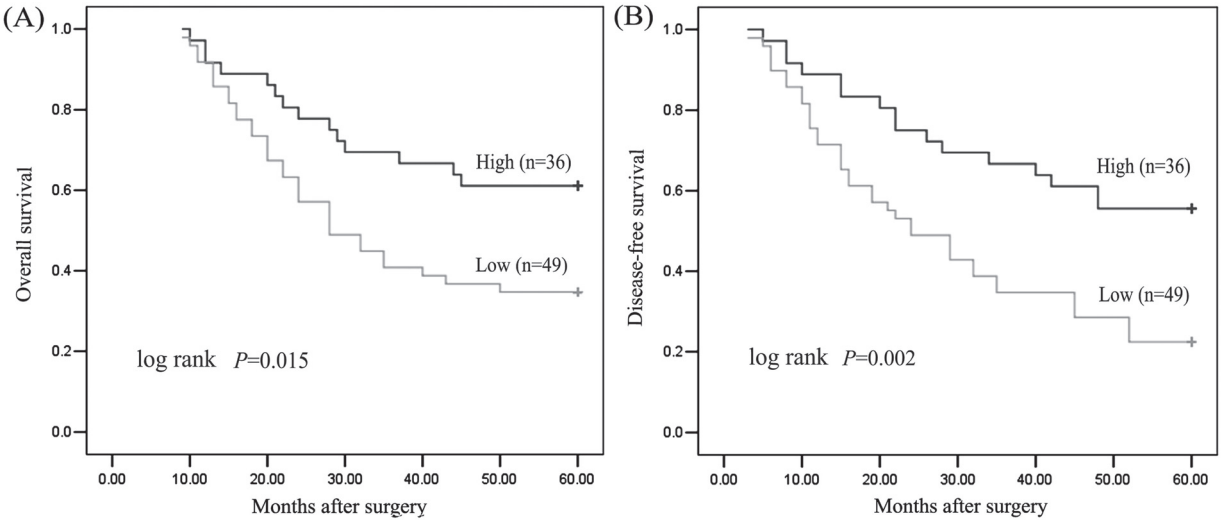

Figure 3. Kaplan-Meier statistical analyses of correlation of fibulin-3 protein levels with overall survival (OS) and disease-free survival (DFS) rates among 85 CRC patients. (A) The 5-year OS rates of CRC patients with low fibulin-3 expression were significantly lower than those with high fibulin-3 expression (*P*=0.015). (B) The 5-year DFS rates of CRC patients with low fibulin-3 expression were significantly lower than those with high fibulin-3 expression (*P*=0.002).

Fibulin-3 protein expression and prognosis in CRC. Since the level of fibulin-3 protein expression was correlated with tumor stage and lymph metastasis, we performed univariate survival analyses to investigate a possible prognostic impact of fibulin-3 in colorectal cancers. As shown in Figure 3, both 5-year overall survival (OS) and disease-free survival (DFS) rates in CRC patients with high fibulin-3 protein level (22/36, 61.1% and 20/36, 55.6%) were superior to those with low fibulin-3 expression (17/49, 34.7% and 11/49, 22.4%), the differences were statistically significant (*P*=0.015 and *P*=0.002). To determine the possibility of downregulation of fibulin-3 as an independent risk factor for poor prognosis, both clinicopathological factors and the level of fibulin-3 protein expression were evaluated by Cox's multivariate analysis. Results showed that tumor stage, regional lymph node metastasis and low level of fibulin-3 expression were independent factors in predicting the adverse OS or DFS for CRC patients (Table 3).

Table 3. Multivariate analysis of clinicopathological factors for overall survival (OS) and disease-free survival (DFS) of 85 patients with CRC

Characteristics	Category	RR (95%CI)	*P*-value
OS			
Age	≥55 *vs* <55 years	1.259 (0.489-3.244)	0.633
Tumor differentiation	Poor *vs* Well/Moderate	2.041 (0.807-5.162)	0.129
Tumor stage	III/IV *vs* I/II	3.000 (1.237-7.273)	0.014*
Lymph metastasis	N_{1-2} *vs* N_0	2.729 (1.132-6.581)	0.024*
Fibulin-3 protein	Low *vs* High expression	2.958 (1.213-7.215)	0.016*
Preoperative serum CEA	≥5.0 *vs* <5.0 ng/mL	1.319 (0.533-3.264)	0.549
DFS			
Age	≥55 *vs* <55 years	1.361 (0.517-3.583)	0.532
Tumor differentiation	Poor *vs* Well/Moderate	2.357 (0.866-6.419)	0.089
Tumor stage	III/IV *vs* I/II	3.091 (1.232-7.765)	0.015*
Lymph metastasis	N_{1-2} *vs* N_0	3.570 (1.403-9.083)	0.006*
Fibulin-3 protein	Low *vs* High expression	4.318 (1.688-11.048)	0.002*
Preoperative serum CEA	≥5.0 *vs* <5.0 ng/mL	1.830 (0.692-4.840)	0.221

RR: Relative risk; 95% CI: 95% confidence interval.
*$P<0.05$

Discussion

Tumor angiogenesis—the ability to form new blood vessels, represents a critical step in tumor development through which the tumor establishes an independent blood supply, consequently facilitating tumor growth, invasion and metastasis [19,20]. This process is regulated through a balance of pro- and anti-angiogenic factors released by the tumor and microenvironment [21], and tumors disrupted the delicate balance to trigger the development of their own blood supply in progression.

Fibulins, a versatile family of extracellular matrix (ECM) glycoproteins, share a stretch of calcium-binding epidermal growth factor-like module followed by a unique C-terminal fibulin type module. They act not only as intermolecular bridges within the ECM to form supramolecular structures, but also as mediators for cellular processes and tissue remodeling [3-5]. So far, dysregulation of these molecules have mostly been found in relation to vasculogenesis and cancer biology [22]. Fibulin-3 was discovered in cultures of senescent human diploid fibroblasts (HDF) established from a patient with Werner syndrome and was found to regulate DNA synthesis [23]. Mutations in fibulin-3 have been linked to heritable macular degeneration which was sometimes characterized by excessive angiogenesis [24], and functional experiment *in vitro* and *in vivo* have shown that, expression of fibulin-3 antagonized endothelial cell activities coupled to angiogenesis [9]. Together with many other results [10-13], fibulin-3 may be a potential tumor suppressor gene which functions as the antagonist of angiogenesis.

In the present study, immunohistochemistry assay showed that, fibulin-3 gene was mostly (57.6%, 49/85) downregulated in CRC, as compared to adjacent normal tissues and benign colorectal adenoma. Furthermore, fibulin-3 gene showed a higher methylation frequency in CRC specimens while rare in non-cancer colorectal tissues, and its promoter methylation state was specifically associated with the low or absent expression of fibulin-3 protein. As DNA methylation is an important regulatory mechanism of gene expression refers to the "epigenetic", hypermethylation will lead to the change of chromatin framework, which represses transcription directly, by inhibiting the binding of specific transcription factors, and indirectly, by recruiting methyl-CpG-binding proteins, thus lead to the down-regulation or silence of tumor suppressor genes (TSGs) and then contribute to carcinogenesis [25,26]. That downregulation of fibulin-3 by promoter methylation confirmed in our results further implies the tumor suppressor role of fibulin-3 in CRC.

This finding was in accordance with other reports in breast, lung cancer and hepatocellular carcinoma (HCC), for example, recently, Nomoto *et al* [13] found fibulin-3 gene was decreased in HCC tumor tissue, using gene expression array, and 24 of 48 HCC samples showed promoter hypermethylation, in the 24 methylated cases, most of the values of fibulin-3 gene expression examined by real-time PCR in tumor tissues were significantly decreased. Meanwhile, 5-aza-2'-deoxycytidine (5-aza-dC), a methyltransferase inhibitor treatment restored the fibulin-3 expression in HCC cell lines, which indicated that promoter methylation directly contributes to the silencing of fibulin-3. Furthermore, analysis of clinically well characterized primary tumors reveals a significant correlation of fibulin-3 hypermethylation or reduced fibulin-3 expression with worse prognosis [10-13]. Fibulin-3 might be useful as molecular markers of these cancers.

Our date demonstrated also that, downrugulation of fibulin-3 protein in CRC was correlated with tumor stage, lymph node metastasis and poor disease-free and overall survival. Recent advances in cancer research have revealed the importance of angiogenesis to cancer progression. In a multivariate analysis of Cox proportional hazard models, we found that low fibulin-3 expression was an independent unfavorable prognostic factor apart from TNM stage and lymph node metastasis. These findings suggest that downregulation of fibulin-3 may influence the patients' prognosis by promoting the angiogenic activity of CRC cells. For CRC patients often suffer asymptomatic metatasis, even after curable surgery in the early stage, it is clearly imperative to develop more effective screening and enhance our ability to predict the disease's course. Currently, molecular markers are becoming the most important complementary prognostic factors for conventional clinicopathological parameters [27], and fibulin-3 may be a new promising biomarker for the prognosis of CRC.

However, several recent studies showed that fibulin-3 was upregulated in glioma [16] and cervical carcinoma [15], and fibulin-3 transfection of FG pancreatic adenocarcinoma cells stimulated orthotopic and metastatic tumor growth *in vivo*, through a stimulation of VEGF production by tumor cells and an increased number of CD31-positive microvessels [14], but endothelial cell proliferation and migration were not altered by fibulin-3, indicating an indirect angiogenic effect. Such contradiction with our results may be due to the fact that fibulin-3 regulates cellular process in a context-specific manner, the different histological types of tumors with different microenvironments may have different expression patterns of fibulin-3, and tumor microenvironment influences the tumor genes to promote angiogenesis and metastasis through the protein-protein interactions [28,29]. For example, tissue inhibitor of metalloproteinases-3 (TIMP-3) is a binding partner of fibulin-3, and their interaction may interfere VEGF binding to VEGF receptor-2, resulting in an inhibition of angiogenesis [30], while fibulin-3 binds EGF receptor (EGFR) in a competitive manner relative to epidermal growth factor (EGF), and activates MAPK and Akt pathways in pancreatic carcinoma cells [31]. Thus, whether fibulin-3 promote angiogenesis and metastasis or not in a certain tumor may depend on its contact with other proteins in the tumor microenvironment, of course, the full mechanism involved still needs further study.

In conclusion, we found that fibulin-3 gene was mostly downregulated in CRC, although widely expressed in non-cancer samples, and DNA methylation is an important regulatory mechanism contributing to fibulin-3 downregulation. Furthermore, the downregulation was correlated with tumor stage, lymph node metastasis and poor disease-free and overall survival, which maybe use as a potential prognostic factor for CRC.

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