Pathol. Oncol. Res. (2012) 18:263–270
DOI 10.1007/s12253-011-9437-0

RESEARCH

CDH13 and FLBN3 Gene Methylation are Associated with Poor Prognosis in Colorectal Cancer

Zhu Wang · Xin Yuan · Nanlin Jiao · Hui Zhu · Youwei Zhang · Jiandong Tong

Received: 27 April 2011 / Accepted: 7 July 2011 / Published online: 28 July 2011

Abstract The aim of this study was to identify potential epigenetic prognostic biomarkers for colorectal cancer (CRC) in the Chinese population. The methylation status of five tumor suppressor genes (*CDH13*, *DLEC1*, *FBLN3*, *hMHL1* and *RUNX3*) was determined using manual microdissection followed by methylation-specific PCR in 85 paired CRC specimens and adjacent normal tissue. The results showed that methylation frequencies in cancerous tissues were 31.8% for *CDH13*, 37.6% for *DLEC1*, 38.8% for *FBLN3*, 22.4% for *hMHL1* and 27.1% for *RUNX3*, all of which were significantly higher than in corresponding normal tissue. Furthermore, *CDH13* methylation was associated with poor differentiation (P=0.019) and tended to be predominant in advanced stages (P=0.084); *FBLN3* methylation was associated with advanced stages (P=0.027) and lymph node metastasis (P=0.029). Accordingly, the methylation status of *CDH13* (P=0.022), *FBLN3* (P=0.008), *CDH13* and/or *FBLN3* (P=0.001) predicted adverse overall survival in CRC, while *hMHL1* methylation showed a protective role in survival (P=0.046). Cox proportional hazard models further indicated that *CDH13* and/or *FBLN3* methylation, but not that of *hMHL1*, was an independent prognostic factor for CRC. In conclusion, we found *CDH13* and *FBLN3* gene methylation are potential biomarkers for poor prognosis in CRC.

Z. Wang · X. Yuan · H. Zhu · Y. Zhang · J. Tong (✉)
Department of Oncology, Yangzhou No.1 People's Hospital,
The second Clinical School of Yangzhou University,
Number 368, Mid Hanjiang Road,
Yangzhou 225009, China
e-mail: tongjd1@yahoo.cn

Z. Wang
e-mail: wangzhu@csco.org.cn

N. Jiao
Department of Pathology, Yijishan Hospital,
Wannan Medical College,
Wuhu 241001, China

Keywords Colorectal cancer · Methylation · Prognosis · Microdissection

Abbreviations

CRC	Colorectal cancer
TSGs	Tumor suppressor genes
CDH13	H-cadherin
DLEC1	Deleted in lung and esophageal cancer 1
FLBN3	Fibulin-3
hMHL1	mutL homolog 1
RUNX3	Runt-related transcription factor 3
MSP	Methylation-specific polymerase chain reaction

Introduction

The worldwide incidence of colorectal cancer (CRC) is on the rise. It is the second-leading cause of cancer-related deaths in Western countries and the third or fourth in China. Even though this disease is surgically curable in the early stages, patients often suffer asymptomatic metastasis, which is associated with a high mortality [1]. Therefore, it is crucial to develop more effective screening and enhance our ability to predict the course of the disease. Currently, the most important conventional prognostic factors are the histological grade and tumor stage at the time of diagnosis (pTNM or Dukes's). In addition to these clinicopathological parameters, molecular markers, such as microsatellite instability (MSI), *KRAS* or *BRAF* mutation status are being sought in CRC [2]. This could lead to improved prognostication and therefore more appropriate adjuvant treatments.

DNA methylation at CpG islands within or near promoter regions has been verified as an important epigenetic regulatory mechanism of gene expression. Hypermethylation leads to a change in chromatin frame-

work, which represses transcription directly by inhibiting the binding of specific transcription factors, and indirectly by recruiting methyl-CpG-binding proteins, thus leading to the downregulation or silencing of tumor suppressor genes (TSGs) and potentially contributing to carcinogenesis and cancer progression [3]. Clinical investigation of the associations between abnormal methylation and cancer diagnosis or prognosis have been conducted in various cancers [4, 5]. DNA methylation has also been studied extensively in CRC, and several methylated loci have been shown to be associated with a worse outcome, including *APC*, *MGMT*, *MINT1*, *MINT31*, $p14^{ARF}$, $p16^{INK4a}$, and *RARβ2* [6–8]. To identify new prognostic biomarkers in the Chinese population, we determined the methylation status of five TSGs, H-cadherin (*CDH13*), deleted in lung and esophageal cancer 1 (*DLEC1*), fibulin-3 (*FBLN3*), mutL homolog 1 (*hMHL1*) and runt-related transcription factor 3 (*RUNX3*) in 85 pairs of CRC and corresponding normal tissues, using microdissection followed by methylation-specific PCR, their clinicopathological significance was further evaluated.

Material and Methods

Study Population

A total of 85 paired CRC specimens and adjacent normal tissue were examined in this study. All samples were formalin-fixed and 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 adenocarcinomas untreated with neoadjuvant radiochemotherapy were recruited for the study. Patients consisted of 52 males and 33 females, with a median age of 59, at stages I (n=12), II (n=28), III (n=44) and IV (n=1 (with liver metastasis)). Histological diagnosis was established on standard H&E stained sections according to the 2000 WHO classification system for tumors of the digestive system, and tumor stage was determined according to the 2002 TNM staging guidelines as 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 informed consent gained from all patients prior to sample examination.

Microdissection and DNA Extraction

Formalin-fixed tissues were manually microdissected using sterile 27-gauge needles with the assistance of a dissection microscope, and referenced to a section stained with H&E, as described previously [9]. Genomic DNA was extracted from microdissected tissue using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Bisulphite Treatment of DNA and Methylation-Specific Polymerase Chain Reaction (MSP)

Extracted DNA was quantified spectrophotometrically, and approximately 1 μg of genomic DNA was bisulphite-treated with EZ-DNA methylation Gold Kit (Zymo Research, Orange, CA, USA), and resuspended in 20 μl of TE buffer. Polymerase chain reaction (PCR) was performed in a 25 μl volume containing 5 μl of DNA template, 10×Buffer, 0.15 mM dNTPs, 0.1 mM of each primer and 0.5U of Ex Taq Hot Start Version (Takara, Shiga, Japan). All primer sequences and amplification conditions are described elsewhere (Table 1) [10–12]. PCR products were identified on a 2% agarose gel stained with ethidium bromide. Lymphocyte DNA, original or methylated in vitro by excess CpG (SssI) methylase (New England Biolabs, Beverly, MA, USA), was used as a positive control Water blank was used as a negative control. To verify the MSP results, stochastic bands from each target were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) gel-purified and directly sequenced on an ABI 3100-Avant DNA sequencer.

Statistical Analysis

Statistical tests were carried out using SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, USA). The methylation index (MI) for each sample was defined as the ratio of the number of methylated genes to the number of genes tested (five in this study), and was compared using analysis of variance (ANOVA). 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. $P<0.05$ was taken as statistically significant.

Results

Methylation Profiles in Primary CRC and Adjacent Normal Colorectal Tissues

Firstly, we employed MSP to assess the promoter methylation status of the 5 TSGs in 85 paired of primary CRC and adjacent normal colorectal tissues. The results showed that methylation frequencies in cancerous tissues were 31.8% (27/85) for *CDH13*, 37.6% (32/85) for *DLEC1*, 38.8% (33/

Table 1 List of MSP primers

GenBank No.	Primers	Sequences (5′–3′)	Products	Location	TM (Cycle)
AB001090	CDH13 (M)	**f:** TCGCGGGGTTCGTTTTTCGC **r:** GACGTTTTCATTCATACACGCG	243 bp	1,402–1,644	56°C(40)
	CDH13 (U)	**f:** TTGTGGGGTTTGTTTTTTGT **r:** AACTTTTCATTCATACACACA	243 bp	1,402–1,644	52°C(40)
AP006309	DLEC1 (M)	**f:** GATTA AGCGATGACGGGATTC **r:** ACC CGACTAATAACGAAATTAACG	193 bp	19,608–19,800	60°C(40)
	DLEC1 (U)	**f:** TGATTATAGTGATGATGG GATTTGA **r:** CCCAAC TAATAACAAAATTAACACC	193 bp	19,607–19,799	60°C(40)
AC010895	FBLN3 (M)	**f:** GTAGTTTTAGGGGATCGTCGC **r:** TCCCCGACACGCTACCTTCG	160 bp	389–548[a]	55°C(40)
	FBLN3 (U)	**f:** GAGTAGTTTTAGGGGATTGTTGT **r:** TCCCCAACACACTACCTTCA	162 bp	387–548[a]	55°C(40)
AB017806	hMHL1 (M)	**f:** TTAATAGGAAGAGCGGATAGC **r:** CTATAAATTACTAAATCTCTTCG	106 bp	1,506–1,611	55°C(40)
	hMHL1 (U)	**f:** TTAATAGGAAGAGTG GATAGTG **r:** TCTATAAATTACTAAATCTCTTCA	107 bp	1,506–1,612	55°C(40)
AL023096	RUNX3 (M)	**f:** ATAATAGCGGTCGTTAGGGCGTCG **r:** GCTTCTACTTTCCCGCTTCTCGCG	115 bp	64,917–65,031	60°C(40)
	RUNX3 (U)	**f:** ATAATAGTGGTTGTTAGGGTGTTG **r:** ACTTCTACTTTCCCACTTCTCACA	115 bp	64,917–65,031	55°C(40)

M methylation; *U* unmethylation; *f* forward; *r* reverse

[a] Location to transcription start

85) for *FBLN3*, 22.4% (19/85) for *hMHL1* and 27.1% (23/85) for *RUNX3*, all of which were significantly higher than in corresponding normal tissue ($P<0.05$, Table 2). Figure 1 shows the typical MSP and sequencing results.

Correlation Between Methylation and Clinicopathological Characteristics in CRC

Next, we analyzed the correlation between the methylation status and the clinicopathological features of CRC. As shown in Table 3, methylation of *CDH13* was more frequent in poorly differentiated tissue than in well/moderately differentiated tissue ($P=0.019$), while *FBLN3* methylation was observed preferentially in stages III/IV ($P=0.027$) or in those exhibiting lymphatic metastasis ($P=0.029$). Although methylation of *CDH13* tended to predominate in stages III/IV and methylation of *hMHL1* occurred predominantly in proximal locations, no statistically significant correlations were found ($P=0.084$ and $P=0.080$, respectively). No other associations were found between the methylation statuses of the five genes and the clinicopathological characteristics, including sex, age and tumor size, but the MI value was significantly higher in older rather than in younger patients ($P=0.037$).

Table 2 Methylation profiles in primary CRC and adjacent normal tissue ($n=85$)

Genes	Methylation frequencies		95% CI, (*P*-value)
	Cancerous tissue n(%)	Normal tissue n(%)	
CDH13	27 (31.8)	8 (9.4)	1.897–10.582 (<0.001)
DLEC1	32 (37.6)	6 (7.1)	3.109–20.327 (<0.001)
FBLN3	33 (38.8)	10 (11.8)	2.277–11.039 (<0.001)
hMHL1	19 (22.4)	5 (5.9)	1.528–12.207 (0.003)
RUNX3	23 (27.1)	7 (8.2)	1.665–10.263 (0.001)

DNA Methylation and Prognosis in CRC

We performed univariate survival analysis to investigate a possible prognostic impact of methylation status of the five genes in CRC. As shown in Fig. 2, the 5-year overall survival (OS) in CRC patients with *CDH13* or *FBLN3* methylation was inferior to those without methylation (mean of 35.1 months (95% CI: 28.5–41.7) vs 45.4 months (95% CI: 40.8–50.0), $P=0.008$, and mean of 36.8 months (95% CI: 30.6–43.0) vs 45.5 months (95% CI: 40.7–50.3), $P=0.022$, respectively). Additionally, methylation of *CDH13* and/or *FBLN3* had a prognostic significance (mean of 36.9 months (95% CI: 31.8–41.9) vs 49.0 months (95% CI: 43.6–54.4), $P=0.001$). *DLEC1* and *RUNX3* methylation had no prognostic significance, while *hMHL1* methylation in CRC was associated with a better outcome (mean of 51.5 months (95% CI: 46.0–57.0) vs 39.4 months (95% CI: 34.9–44.0), $P=0.046$).

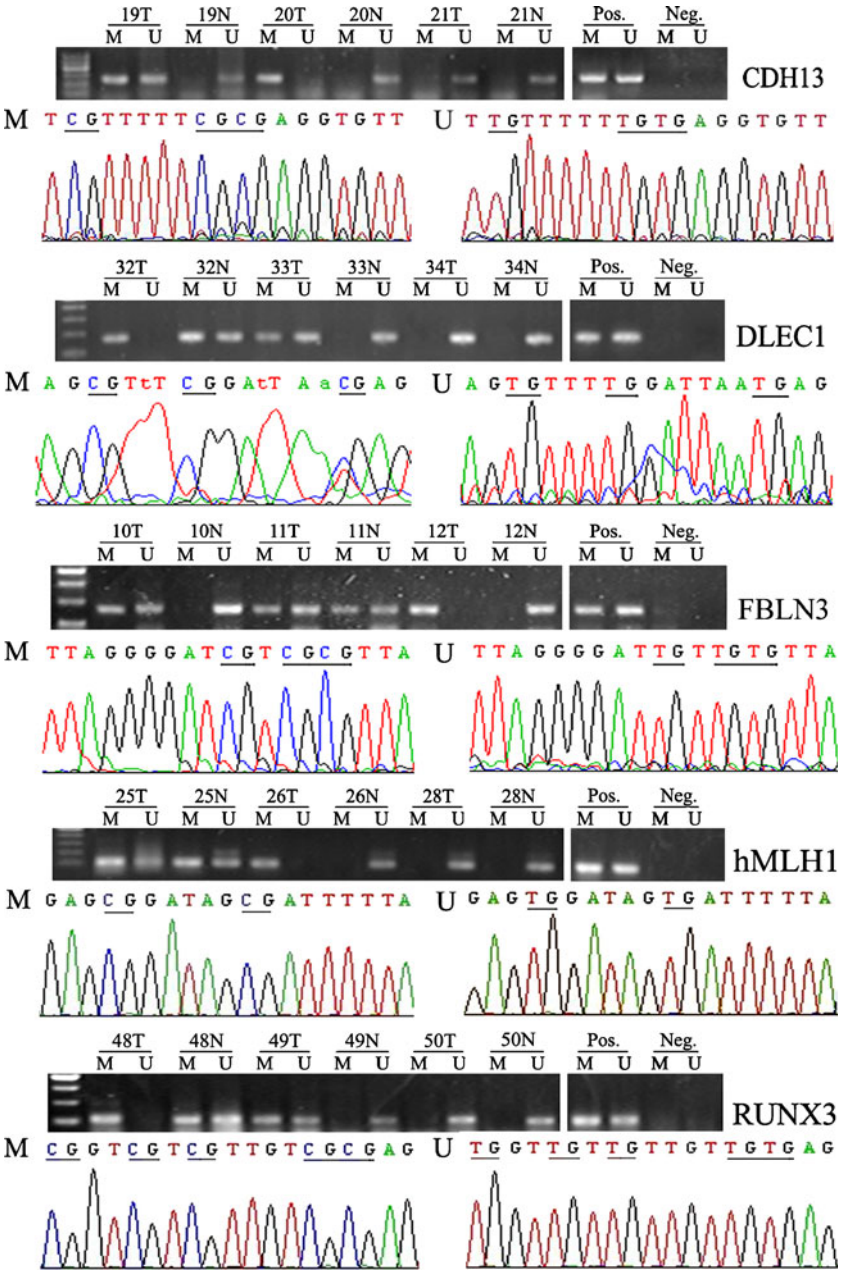

Fig. 1 Methylation profiles in matched CRC and adjacent normal tissues. **a** Typical agarose gel electrophoresis of MSP results in tissue samples. T, tumor tissues; N, adjacent normal lung tissues. Lymphocyte DNA, original or methylated in vitro by excessive CpG (SssI) methylase, was used as a positive control. Water blank was used as a negative control. **b** MSP products were directly sequenced and confirmed. Methylated cytosines (C) are not converted to uracil (T) and remain as C

Further, multiple Cox regression analysis was used to verify whether the investigated variables, including aberrant methylation, are valid predictors of outcome after adjustment for potential confounding cofactors. Results showed that tumor stage, regional lymph metastasis and methylation of *CDH13* and/or *FLBN3* were independent factors in predicting the adverse OS for CRC patients (Table 4).

Discussion

Aberrant DNA methylation is useful as a molecular marker in human cancers. In the current study, we determined the promoter methylation status of five TSGs and identified their associations with the prognosis of CRC patients, using manual microdissection/MS-PCR analysis. Microdissection techniques have addressed the dilemma in cancer research that genomic DNA is susceptible to contamination by non-neoplastic cells, such as fibroblasts vascular endothelial cells and other mesenchymal cells [13, 14]. Thus, the mask of tumor specific alterations by heterogeneity may be decreased and the methylation profile of cancerous cells will be precisely illustrated.

Five genes potentially important in CRC pathogenesis were selected based on the literature, and were shown here to undergo sensitive and specific methylation in CRC tissues, compared to the normal mucosa tissues [15–17]. Excluding the *FBLN3* gene, all the others have been

Table 3 Association between DNA methylation in CRC and clinicopathological features

Characteristics	Methylation frequency, *n* (%)					Median MI (IQR)
	CDH13	DLEC1	FBLN3	hMHL1	RUNX3	
Gender						
Male (*n*=52)	18 (34.6)	23 (44.2)	21 (40.4)	9 (17.3)	12 (23.1)	0.2 (0.2–0.4)
Female (*n*=33)	9 (27.3)	9 (27.3)	12 (36.4)	10 (30.3)	11 (33.3)	0.2 (0.2–0.4)
Age						
<55 (*n*=24)	6 (25.0)	6 (25.0)	8 (33.3)	4 (16.7)	4 (16.7)	0.2 (0.2–0.2)
≥55 (*n*=61)	21 (34.4)	26 (42.6)	25 (40.9)	15 (24.6)	19 (31.2)	0.4 (0.2–0.4)*
Tumor site						
Proximal (*n*=22)	9 (40.9)	10 (45.5)	9 (40.9)	8 (36.4)	6 (27.3)	0.4 (0.2–0.4)
Distal (*n*=33)	10 (30.3)	13 (39.4)	11 (33.3)	6 (18.2)	10 (30.3)	0.2 (0.2–0.4)
Rectum (*n*=30)	8 (26.7)	9 (30.0)	13 (43.3)	5 (16.7)	7 (23.3)	0.2 (0.2–0.4)
Tumor size						
≤5 cm (*n*=48)	14 (29.2)	15 (31.3)	18 (37.5)	9 (18.8)	13 (27.1)	0.2 (0.2–0.4)
>5 cm (*n*=37)	13 (35.1)	17 (46.0)	15 (40.6)	10 (27.1)	10 (27.0)	0.4 (0.2–0.4)
Cellular differentiation						
Well (*n*=19)	4 (21.1)	6 (31.6)	6 (31.6)	3 (15.8)	5 (26.3)	0.2 (0.2–0.4)
Moderate (*n*=37)	9 (24.3)	16 (43.2)	14 (37.8)	10 (27.0)	8 (21.6)	0.2 (0.2–0.4)
Poor (*n*=29)	14 (48.3) *	10 (34.5)	13 (44.8)	6 (20.7)	10 (34.5)	0.4 (0.2–0.4)
Stage						
I (*n*=12)	3 (25.0)	5 (41.7)	4 (33.3)	2 (16.7)	4 (33.3)	0.2 (0.2–0.4)
II (*n*=28)	6 (21.4)	11 (39.3)	6 (21.4)	7 (25.0)	5 (17.9)	0.2 (0.2–0.4)
III/IV (*n*=45)	18 (40.0)	16 (35.6)	23 (51.1) *	10 (22.2)	14 (31.1)	0.4 (0.2–0.4)
Lymph metastasis						
N_0 (*n*=41)	10 (24.4)	16 (39.0)	11 (26.8)	9 (22.0)	9 (22.0)	0.2 (0.2–0.4)
N_1/N_2 (*n*=44)	17 (38.6)	16 (36.4)	22 (50.0) *	10 (22.7)	14 (31.8)	0.4 (0.2–0.4)

MI methylation index; *IQR* interquartile range

*$P<0.05$

previously reported to be frequent methylated and functionally important in CRC. The *CDH13* gene product belongs to the cadherin family of cell surface glycoproteins responsible for selective cell recognition and adhesion [18], while the *DLEC1* gene encodes a 1755-amino-acid protein with no significant homology to known proteins or conserved domains, which has been verified as a *bona fide* tumor suppressor gene in cell cycle regulation [19]. *RUNX3* belongs to the runt domain family of transcription factors that respond to growth, differentiation and apoptosis signals induced by the transforming growth factor (TGF-β) pathway [20]. Finally, *hMHL1* is a member of the family of mismatch repair (MMR) genes, whose functional inactivation results in multiple replication errors in repetitive DNA sequences [21]. Their methylation frequencies in our study are in the range of those reported in previous investigations.

FBLN3 gene, also known as *EFEMP1* (epidermal growth factor-containing fibulin-like extracellular matrix protein 1), is a member of the fibulin gene family that consists of seven extracellular matrix (ECM) proteins [22]. Functional experiments in vitro and in vivo have shown that *FBLN3* acts as a potential tumor suppressor gene, for its expression antagonizes endothelial cell activities coupled to angiogenesis [23], and its downregulation due to promoter methylation has been found in breast [24], lung [25] and hepatocellular carcinoma (HCC) [26]. Nomoto et al. [26] recently reported that *FBLN3* gene expression was reduced in HCC tumor tissue, and 50% of samples examined showed promoter hypermethylation, clinical analysis also revealed a significant correlation of *FBLN3* methylation or reduced *FBLN3* expression with worse HCC prognosis. Our results showed a high frequency of *FBLN3* methylation in CRC specimens compared with normal colorectal tissues. This is the first report of *FBLN3* aberrant methylation in CRC.

Furthermore, *FBLN3* gene methylation was associated with advanced CRC and lymph node metastasis of CRC patients. *CDH13* methylation was associated with poor differentiation, and showed predominance in advanced

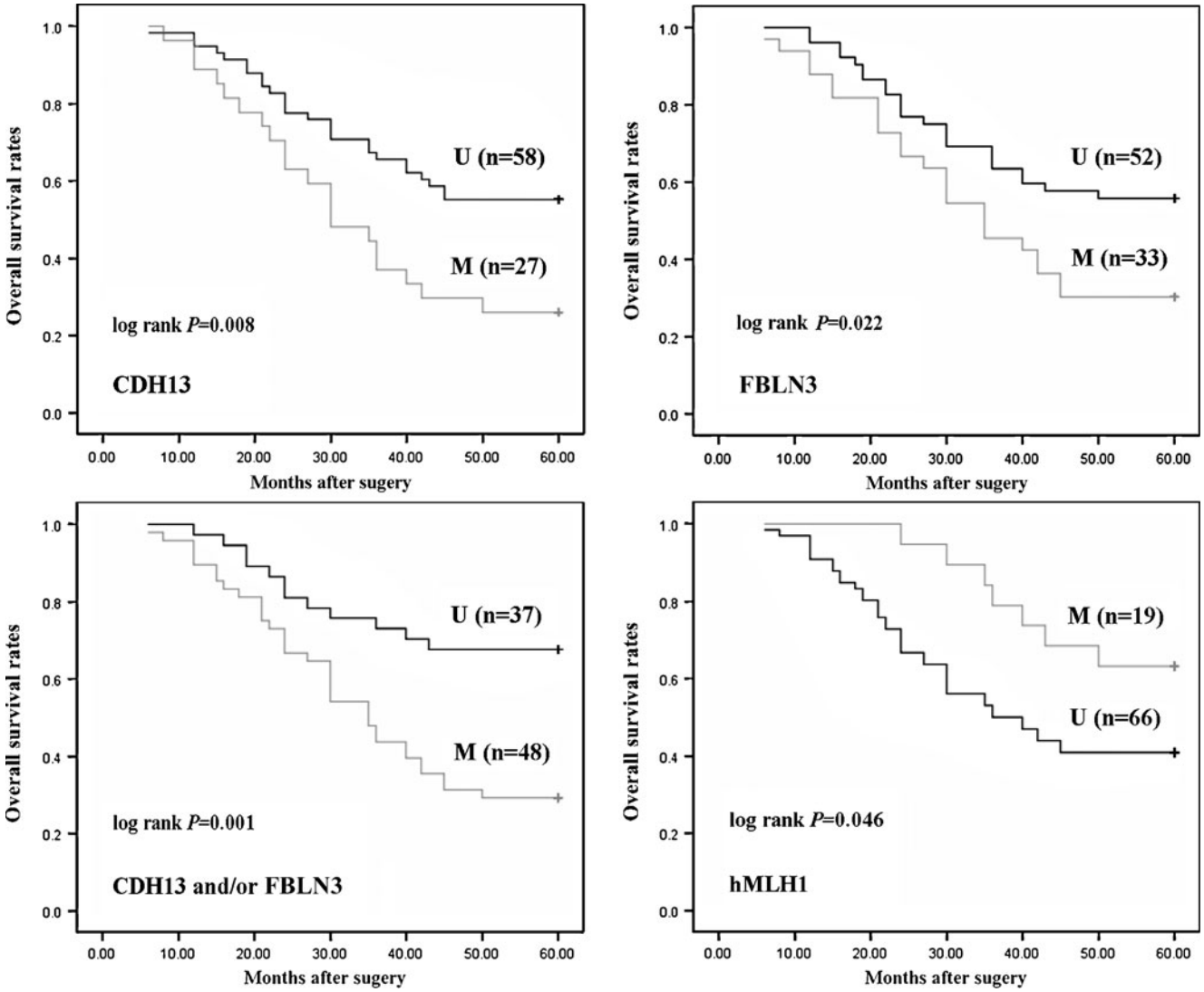

Fig. 2 Correlation between the methylation status of *CDH13* (A), *FBLN3* (B), *CDH13* and/or *FBLN3* (C), *hMHL1* (D) and 5-year overall survival rate of 85 patients using Kaplan-Meier analysis. The *survival curves* show the patients with all stages of disease. *M* methylated case; *U* unmethylated case

stages (not statistically significant), suggesting the involvement of these two genes in the progression of CRC. As expected, *CDH13* and *FBLN3* gene methylation were both correlated with adverse 5-year OS, also in combination, patients with methylation of CDH13 and/or fibulin-3 had a prognostic significance. In a multivariate analysis of Cox proportional hazard models, we verified that methylation of *CDH13* and/or *FBLN3* was an independent unfavorable

Table 4 Multivariate analysis of clinicopathological factors for the overall survival (OS) of 85 patients with CRC

Characteristics	Category	RR (95%CI)	*P*-value
Age	≥55 vs <55 years	1.259 (0.489–3.244)	0.633
Tumor site	Proximal vs Distal/Rectum	1.750 (0.646–4.744)	0.298
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	$N_{1\text{-}2}$ vs N_0	2.729 (1.132–6.581)	0.024*
CDH13 and/or *FBLN3* methylation	Methylation vs Unmethylation	5.060 (2.000–12.798)	<0.001*
hMHL1 methylation	Methylation vs Unmethylation	0.404 (0.141–1.158)	0.086
Preoperative serum CEA	≥5.0 vs <5.0 ng/mL	1.319 (0.533–3.264)	0.549

RR relative risk; 95% CI: 95% confidence interval

*$P<0.05$

prognostic factor apart from TNM stage and lymph node metastasis. We supposed that silencing of these two genes by promoter methylation may influence prognosis by promoting metastasis and angiogenesis, respectively.

It is of note that methylation of *hMHL1* showed predominance in proximal locations (not statistically significant), and was correlated with improved 5-year OS. This corresponds with previous studies reporting that *hMHL1* promoter methylation is found in most sporadic colorectal cancers with high microsatellite instability (MSI-H) [27, 28]. As DNA MMR gene inactivation is one of the main factors leading to general genome instability, the methylation status of *hMHL1* and the consequences of MSI have been considered as crucial events leading to the development of CRC [29]. The prevalent characteristics of MSI-H CRC with *hMHL1* methylation include female preponderance, proximal tumor location, older age and protective roles in survival [30, 31]. Our results confirmed that the methylation status of *hMHL1* gene predicts a better outcome, but it is not an independent prognostic factor, as shown by Cox proportional hazard models, which may be due to effects of other factors, such as patients with *hMHL1* gene methylation was more sensitive to 5-FU chemotherapy [32].

In addition, *DLEC1* and *RUNX3* gene methylation showed no association with the clinicopathological characteristics and 5-year OS. Although no associations were found between the methylation status of the five genes and age, the MI value was significantly higher in older than in younger patients, suggesting age-related methylation, a universal phenomenon in epithelial malignancies [33], exists in CRC.

In conclusion, microdissection followed by methylation-specific PCR may be a precise method in the determination of methylation profile, and we found *CDH13* and *FBLN3* gene methylation are associated with poor prognosis in CRC.

Acknowledgements We are grateful for the participation of our patients and healthy donors, without which our study would not have been possible. The valuable help from the Departments of Pathology of Yijishan Hospital is also greatly appreciated.

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