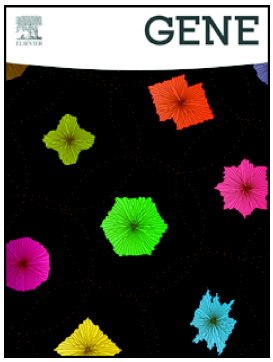

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DNA methylation of *CMTM3*, *SSTR2*, and *MDFI* genes in colorectal cancer

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Abstract

Colorectal cancer (CRC) is increasingly common worldwide, including in China. Therefore, there is an increasing need to detect CRC at an early stage and to discover and evaluate diagnostic and prognostic biomarkers. DNA methylation of genes in CRC is a potential epigenetic biomarker for the early detection of CRC. This study was performed to analyze the methylation frequency of six candidate genes, *CMTM3*, *SSTR2*, *MDFI*, *NDRG4*, *TGFB2*, and *BCL2L11*, in fresh-frozen CRC tissues and adjacent normal colorectal tissues, from 42 patients with CRC. DNA isolation, bisulphite modification, and pyrosequencing were performed. The sensitivity, specificity, and the area under the receiver operator characteristic (ROC) curve (AUC) were evaluated to determine whether these genes showed any associations with tumor grade, stage, or diagnostic features. Among the tested genes, three genes, *CMTM3*, *SSTR2*, and *MDFI* were significantly methylated in CRC tissues when compared with adjacent normal colorectal tissues. The ROC analysis showed that a multigene model, including *CMTM3*, *SSTR2*, and *MDFI*, had a sensitivity of 81% and a specificity of 91% with an AUC value of 0.92. The findings of this study have shown that DNA methylation of the genes, *CMTM3*, *SSTR2*, and *MDFI* should be studied further with a view to determining their potential role as biomarkers for CRC.

Keywords: colorectal cancer, CRC, *CMTM3*, *MDF1*, *SSTR2*, DNA methylation

1. Introduction

Worldwide, colorectal cancer (CRC) is the third most common cancer in men and women, with an estimated 134,490 new cases and 49,190 deaths from CRC in the USA in 2016 [1]. The incidence of CRC is increasing in China [2] and now involves younger individuals [3].

Etiological studies have demonstrated that the interaction between genetic and epigenetic changes is associated with the development of CRC [4, 5]. Societal factors such as low levels of vegetable, fruit and fish consumption, coupled with a high dietary intake of meat and fat, are also correlated with an increased risk of CRC in various geographical areas [6-8]. The typical development and progression of CRC occur over years and even decades, with the accumulation of several genetic events by the time the tumor becomes invasive and metastasizes [9]. As an example of the multi-stage and multi-gene progression of CRC, mutation of the *APC* gene is thought to initiate CRC [10, 11]; mutation of the *P53* gene is involved in the malignant transformation of CRC [12]. Also, the majority of primary CRCs have been shown to harbor several aberrations at the chromosomal level [13].

Recently, epigenetic modification has become a focus of scientific and clinical research in oncology and has been shown to be involved in the progression of malignant tumors, including CRC [14-16]. DNA methylation, one of the most important epigenetic changes, occurs in the cytosine of CpG dinucleotides, is associated with the inactivation of key genes, and appears to cooperate with the

genetic alterations required to drive the initiation and progression of CRC [17, 18].

The transforming growth factor-β (TGF-β) signaling pathway [19], Bcl-2 protein family [20], PI3K-Akt pathway [21], and Wnt/β-catenin pathway [22] have been shown to have a role in tumorigenesis in CRC. These signaling pathways regulate important cellular processes including apoptosis, cell proliferation, and inflammation. The *TGFB2* gene is a protein-encoding gene for the TGF-β superfamily, inducing apoptosis via the TGF-β signaling pathway [19]. Bcl-2 like 11 (*BCL2L11*), also known as Bim protein, is a member of the Bcl-2 protein subfamily and is involved in regulating the TGF-β pathway [23]. CKLF-like MARVEL transmembrane domain-containing member 3 (*CMTM3*) can induce apoptosis by activation of caspase-3 [24]. N-Myc downstream-regulated gene family 4 (*NDRG4*) suppresses tumor via the PI3K-Akt signaling pathway [25]. Somatostatin receptor 2 (*SSTR2*) is reported to induce cell apoptosis by promoting the plasma membrane accumulation of β-catenin [26, 27]. MyoD family inhibitor 1 (*MDFI*) modifies the expression of β-catenin through interaction with axin [28, 29].

The purpose of this study was to analyze the methylation frequency of six candidate genes, *CMTM3*, *SSTR2*, *MDFI*, *NDRG4*, *TGFB2*, and *BCL2L11*, in fresh-frozen CRC tissues and adjacent normal colorectal tissues, from 42 patients with histologically confirmed primary CRC who underwent surgical tumor resection.

2. Materials and methods

2.1 Tissue samples

The study included 42 patients who had a histological diagnosis of primary colorectal cancer (CRC) and who had surgical resection of the primary tumor. The cancerous and normal adjacent colorectal tissue samples were collected from the Department of Gastrointestinal Surgery in the Affiliated Hospital of Ningbo University from June 2012–April 2013. All the CRC tissue diagnoses were performed by experienced pathologists. Tumor tissues and the corresponding normal colorectal tissues from each patient were sampled by an experienced pathologist following surgical resection. Normal adjacent tissue was sampled at least 5 cm beyond the macroscopically identified tumor. All the tissue samples were stored in liquid nitrogen at -80^{o}C immediately after sampling. A photomicrograph of a representative tissue section of CRC and normal colorectal tissue, stained with hematoxylin and eosin (H&E), is shown in Figure 1. None of the patients in this study had a history of preoperative chemotherapy or radiation therapy. Before surgery and tissue collection, all patients provided informed written consent for the surgical procedure, tissue sampling, and participation in the study. The clinicopathological characteristics of the patients in this study are shown in Table 1.

2.2 DNA isolation

Genomic DNA from 42 tumors and 42 paired adjacent normal colorectal tissue samples were isolated by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. DNA concentration and quality were determined by the ultramicro nucleic acid ultraviolet tester (NanoDrop 1000, Wilmington, DE, USA).

2.3 Bisulphite modification

Eluted DNA was bisulphite-converted with ZYMO EZ DNA Methylation-Gold Kit according to the manufacturer's protocol (Zymo Research, Orange, CA, USA). The bisulphite-modified DNA was resuspended in 10 μl of TE buffer for the following pyrosequencing assay.

2.4 Pyrosequencing assay

Polymerase chain reaction (PCR) was performed to amplify a fragment in the gene promoter (Qiagen, Hilden, Germany) in a 20 μl volume containing 0.3 μM each of forward and reverse primers. Pyrosequencing primer was subsequently utilized to analyze the methylation levels of targeted CG sites. Table 2 shows all the primers designed by PyroMark Assay Design software (Qiagen, Hilden, Germany). Pyrosequencing analysis was carried out by Q24 machine and reagents (Pyromark Gold Q24 Reagents; Qiagen) according to the manufacturer's instructions.

2.5 Extraction of TCGA data

TCGA data of *CMTM3*, *SSTR2* and *MDFI* methylation in 395 CRC patients and 45 normal control tissues were downloaded to analyze methylation difference between CRC and noncancerous tissues. The correlation of *CMTM3*, *SSTR2* and *MDFI* methylation and gene expression was generated from cBioPortal for Cancer Genomics (http://cBioPortal.org) (PubMed ID: 23550210). The cBioPortal for Cancer Genomics is an open-access bio-database, providing visualization and analyzing tool for large-scale cancer genomics data sets. This online portal collected 382 CRCs methylation data and gene expression data from TCGA.

2.6 Statistical analyses

Statistical analysis was performed by applying the SPSS statistical package (version 16.0; SPSS, Chicago, IL, USA). Paired t-tests were used to compare the differences in methylation between the CRC and normal control tissue groups. The variations in methylation in different tumor stages were analyzed using one-way ANOVA. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used to obtain the highest sensitivity and specificity. *P*-values were adjusted for patient age and gender. A P value < 0.05 was considered to be statistically significant. The results were presented using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1 Methylation levels of six genes in colorectal cancer (CRC)

To determine the methylation status of the six genes, *CMTM3*, *SSTR2*, *MDFI*, *NDRG4*, *BCL2L11*, and *TGFB2*, tissue samples of colorectal cancer (CRC) and adjacent normal colorectal tissues were analyzed from the 42 patients in this study using the bisulphite pyrosequencing assay. There were a total of 8, 6, 7, 5, 9, and 7 tested CG sites for *CMTM3*, *SSTR2*, *MDFI*, *NDRG4*, *BCL2L11*, and *TGFB2*, respectively (Figure S1). The mean methylation frequency of tested CGs was analyzed to evaluate the methylation levels of each gene.

The results showed that the mean methylation levels of *CMTM3*, *SSTR2*, and *MDFI* were significantly elevated in CRC tissues compared with adjacent normal tissues (*CMTM3*: adjusted *P* value = 1.4E-04; *SSTR2*: adjusted *P* value = 4.7E-03; *MDFI*: adjusted *P* value = 4.7E-04). The comparison of the difference in the mean methylation frequencies of *TGFB2*, *BCL2L11*, and *NDRG4* between the CRC tumor tissue and normal colorectal tissue did not reach significance. The comparison, of all six genes, between the CRC tissues and normal colorectal tissues are shown in Figure 2. We further extracted methylation data of these three genes (*CMTM3*, *SSTR2* and *MDFI*) among 395 CRC tissues and 45 normal control tissues from (https://genome-cancer.ucsc.edu/). The results of the data mining confirmed that higher methylation level of *CMTM3*, *SSTR2* and *MDFI* in CRC tissues when

compared with normal controls (*CMTM3*: *P* = 7.06E-57, *SSTR2*: *P* = 2.52E-66, *MDFI*: *P* = 3.43E-91, Figure S2).

3.2 Methylation levels of six genes in CRC and clinicopathologic parameters

As shown in Table 1, the methylated *TGFB2* gene was associated with the clinical stage of CRC (*P* = 0.02), the methylated *NDRG4* was associated with cancer location (*P* = 0.009), and there was a significant difference in the methylated *CMTM3* gene with tumor type, with a distinction between adenocarcinoma and mucinous adenocarcinoma (*P* = 0.04). However, there was insufficient evidence to support a significant association between the methylation levels of six genes and clinicopathological features, such as gender, age, lymph node status, metastasis status, tumor differentiation status (grade), and tumor size. There was no correlation between gene methylation with the serum biomarker levels of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). The correlation between clinicopathological features and the methylation frequency of these six genes was not significant in subgroup analysis for gender (*data not shown*).

3.3 The reverse correlation of DNA methylation and expression

Considering the resultant of reduction of expression with gene hypermethylation, we aimed to identify the relationship of *CMTM3*, *SSTR2* and *MDFI* methylation and expression. Therefore, an *in silico* analysis of data retrieved from cBioPortal was performed. A total of 382 CRCs methylation data and corresponding gene expression data were collected from TCGA in this online portal and the results of cBioPortal identified the negative correlation of *CMTM3*, *SSTR2* and *MDFI* methylation and expression in CRCs (Figure S3).

3.4 Receiver operator characteristic curve (ROC) analysis

As shown in Figure 3, the results showed that the AUC values of *CMTM3*, *SSTR2* and *MDFI* were 0.79, 0.74 and 0.80, respectively. Based on a logistic regression model, the results showed four panels (*CMTM3 + SSTR2*, *CMTM3 + MDFI*, *SSTR2 + MDFI* and *CMTM3 + SSTR2 + MDFI*) that separately produced the AUC values of 0.88, 0.82, 0.90, and 0.9, which were higher than for each single gene. Furthermore, the ROC analysis achieved a sensitivity of 81% when all three genes were applied, which was greater than the sensitivity of any of the three individual genes.

4. Discussion

Aberrant promoter hypermethylation constitutes an alternative to coding-region mutation for loss of gene function, which is a crucial event in the initiation and progression of colorectal cancer (CRC) [30-32].

In this study, the methylation of the genes *CMTM3*, *SSTR2*, *MDFI*, *NDRG4*, *TGFB2*, and *BCL2L11* were analyzed, as well as the correlation between these methylation changes and the clinicopathological parameters in 42 patients with CRC. High levels of promoter methylation of *CMTM3*, *SSTR2* and *MDFI* were found in DNA from tissue samples of CRC, and generally low levels were detected in DNA from matched normal colorectal tissue samples ($P < 0.001$). There was no significant correlation between the three highly methylated genes and the clinical stage of CRC, suggesting that the aberrant methylation of these genes may play a role in the initiation of CRC rather than in its progression. This hypothesis requires further study. Interestingly, the methylation difference of *TGFB2* and *NDRG4* between CRC and matched normal tissues was not statistically significant, whereas methylated *TGFB2* and *NDRG4* were found to be associated with disease stage and tumor location, respectively. TGF-β signaling pathway has bidirectional effects, acting as a tumor suppressor during early tumorigenesis, but stimulating cancer progression in later tumorigenesis [33]. The loss of TGF-β signaling in the early stage of patients and the overexpression of TGF-β in many late stage and metastatic patients suggested that the effect of TGF-β, as well as the regulation mechanism of TGF-β, could change in different stages of cancer [34]. However, it is also needed a well-designed large

sample size study to justify the differential methylation level of *TGFB2* in the different stages of CRC. In the current study, the methylation difference of *NDRG4* was observed between 26 colon cancer and 16 rectum cancer. While previous study has reported differential methylated gene among four different sublocation of CRC based on a large sample size study [35], suggesting that this finding needs for further confirmation in the future.

The *CMTM3* gene belongs to the CKLF-like MARVEL transmembrane domain (*CMTM*) family with important functions in the immune system, male reproductive system, and tumorigenesis [36-38]. Previous studies have reported the common observation of down-regulation of CMTM3 in various human cancers, including hepatocellular carcinoma [39], prostate cancer [40], and have justified the hypermethylation of *CMTM3* resulting in loss of expression in oral squamous cell cancer [41], laryngeal squamous cell carcinoma [42]. It has been well documented that *CMTM3* involved in tumorigenesis from multiple dimensions. Re-expression of CMTM3 in human cancer cells can not only inhibits cell growth by cell cycle arrest [43] , but also can causes apoptosis with capase-3 reactivation [24]. Whereas, the overexpression and knockdown of CMTM3 in hepatocellular carcinoma cells [39] and gastric cancer cells [44] from both positive and negative aspects have confirmed that CMTM3 can inhibit the process of EMT through JAK2/STAT3/Twist1 signaling pathway, blocking the invasion and migration of cancer cells.

Somatostatin receptor (*SSTR*) is a family of G-protein coupled plasma membrane receptors of somatostatin peptides [45]. Five SSTR subtypes have been characterized:

SSTR1, *SSTR2*, *SSTR3*, *SSTR4* and *SSTR5*, among which *SSTR2* is widely distributed in normal tissues. Somatostatin and its analogues exert anti-cancer effects attributing to bind with SSTR activating intracellular pathways to inhibit proliferation and induce cell apoptosis [46-48]. However, unsatisfactory therapeutic results of somatostatin analog treatment have been frequently observed with the loss of expression of SSTRs, especially SSTR2 in some human cancers [49]. Besides, the silencing of SSTR2 correlated with histological differentiation, depth of invasion, TNM stage, lymph node metastasis and poor overall survival of most of these cancers [49, 50] .

MDFI has a highly conserved cysteine-rich domain, termed the I-mfa domain [51]. *MDFI* is more than a negative regulator of myogenic regulatory factors, which also serves as tumor suppressor gene involving the tumorigenesis [52]. *MDFI* inhibit the Wnt/beta-catenin signaling through interaction with the axin complex and lymphocyte enhancer factor (LEF) [28, 29, 52]. As an important negative regulator of Wnt pathway, the loss of *MDFI* associated with human breast cancer and myeloid neoplasm [53]. Methylation of *CMTM3*, *SSTR2* and *MDFI* had been described previously in gastric cancer, non-small cell lung cancer (NSCLC), and renal cell carcinoma [24, 43, 54]. The findings of this study are supported by the published findings on other types of cancer, but this study has shown that hypermethylation of these genes also exists in CRC.

There have been previous studies that have identified significant methylation differences between early and advanced stages of CRC [55, 56], and provided evidence that some methylated genes may drive the initiation and progression of CRC

[5, 57]. Methylated genes have also been detected in plasma or in stool samples from patients with CRC [58, 59]. The detection of gene hypermethylation in stool samples has implications for the diagnosis of early-stage CRC and may lead to the development of screening method for patients who are at high-risk for developing CRC.

Previous studies have shown low sensitivity or specificity of gene methylation detection in CRC, but in this study, the methylation of *CMTM3*, *SSTR2*, and *MDFI* have been shown to be specific biomarkers for CRC, in the patients studied. This specificity has been supported by multivariate logistic regression analysis, ROC and AUC analysis. Previous studies have employed multivariate logistic regression analysis models for multigene methylation analysis, including in medulloblastoma and invasive bladder cancer [60-62]. The finding in this study of the high sensitivity of quantitative promoter methylation analysis for the detection of CRC supports the potential diagnostic role of emerging epigenetic markers for CRC and other cancers. Clearly, due to the limited amount of samples in the current study, it is eager to require further studies to determine whether these potential gene methylation biomarkers can be detected in serum or exfoliated cell samples or stool samples.

In conclusion, the findings of this study have shown that the combination of *CMTM3*, *SSTR2*, and *MDFI* gene methylation may be an epigenetic biomarker for the early stages of CRC. DNA methylation of the genes, *CMTM3*, *SSTR2*, and *MDFI* should be studied further with a view to determining their potential role as non-invasive diagnostic biomarkers for CRC.

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Competing financial interests

None of the authors have any commercial or other associations that might pose a conflict of interest.

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Figure legends

Figure 1: Photomicrographs of hematoxylin and eosin (H&E) stained tissue sections of colorectal cancer (CRC) and adjacent normal colorectal tissue

A (x 40) and B (x 200): Photomicrographs of normal colorectal tissue adjacent to colorectal cancer (CRC). Note the well-organized benign glandular structures typical of normal colonic mucosa (H&E).

C (x 40) and D (x 200): Photomicrographs of colorectal cancer (CRC) tissue showing irregular, malignant glands, necrosis, inflammation, and foci of tumor invasion (H&E).

Figure 2: Comparison of average methylation levels of six genes between samples of colorectal cancer (CRC) and control normal adjacent colorectal tissue*

*ns: not significant

Figure 3: Receiver operator characteristic (ROC) curves for the methylation of *CMTM3*, *SSTR2* and *MDFI*

Tables

Table 1: Characteristics of the 42 patients with colorectal cancer (CRC) included in the study

Characteristics		N	*CMTM3_P*	*SSTR2_P*	*MDFI_P*	*NDRG4_P*	*BCL2L11_P*	*TGFB2_P*
Gender	Male / Female	28/14	*	*	*	*	*	*
Age	⩽60 / >60	16/26	*	*	*	*	*	*
Stage[#]	Ⅰ / Ⅱ /Ⅲ/Ⅳ	7/14/13/8	*	*	*	*	*	0.02[#]
Lymph metastasis	Yes / No	21/21	*	*	*	*	*	*
Distant metastasis	Yes / No	8/34	*	*	*	*	*	*
CEA	⩾5.0 ng/ml / <5.0 ng/ml	14/28	*	*	*	*	*	*
CA19-9	⩾37U/ml / <37U/ml	13/29	*	*	*	*	*	*
Tumor location	Colon / Rectum	26/16	*	*	*	0.009	*	*
Differentiation	Poor / Moderate	10/32	*	*	*	*	*	*
Tumor size	<5 cm / ⩾5 cm	28/14	*	*	*	*	*	*

Histological classification	Adenocarcinoma / Mucinous adenocarcinoma	40/2	0.04	*	*	*	*	*

* Ns: not significant.

[#]: The one-way ANOVA method was used to analyze the difference in methylation between the different stages of colorectal cancer (CRC).

Table 2: Polymerase chain reaction (PCR) sequences and sequencing primers

Gene	Forward primer (5’→3’)	Reverse primer (5’→3’)	Sequencing primer (5’→3’)	Tm
CMTM3	Biotin-AGATAGTTTTTTTGGATAGGGGTAG	CCACCTTCTTCTTCACCCTAAT	CTTCTTCTTCACCCTAATT	54.8 ºC
SSTR2	Biotin-AGGGTAGAGGAGTTAGGAATTT	ACCCCTCACCTTTACTTTTC	ACCCAACCACTATCCC	54.8 ºC
MDFI	Biotin-GTGTTAGGTTTTTGGTTGGGTTAAG	CCCCTTCCTTCTTCCTTCTCT	CTTCCCTAACCCACC	54.8 ºC
NDRG4	Biotin-AGGGTTGGGGGTTTTAGA	CACCCTCTACCAAAAACTCAAAACTCAATT	GGGGTTTTAGAGTGTAT	55.4 ºC
BCL2L11	Biotin-GAGGAAGTTGTTGGAGGAGAAT	ACCCTACCCATCCCTATAC	ATCCCCAAACCCAAT	54.8 ºC
TGFB2	Biotin-GAGTAATTTTAAGTTGGGGAGAAGTTAGT	ACTCCAAACCCCAACCCAACCA	CCAACCCAACCACAA	53.2 ºC

Table 3: Diagnostic values of single and combined methylation markers in colorectal cancer (CRC)

Biomarker	Sensitivity (%)	Specificity (%)	AUC
CMTM3	71.4	85.7	0.80
SSTR2	42.9	97.6	0.74
MDFI	66.7	95.2	0.80
CMTM3 + SSTR2	76.2	92.9	0.88
CMTM3 + MDFI	73.8	92.9	0.82
SSTR2 + MDFI	81.0	91.0	0.90
CMTM3 + SSTR2 + MDFI	81.0	91.0	0.92

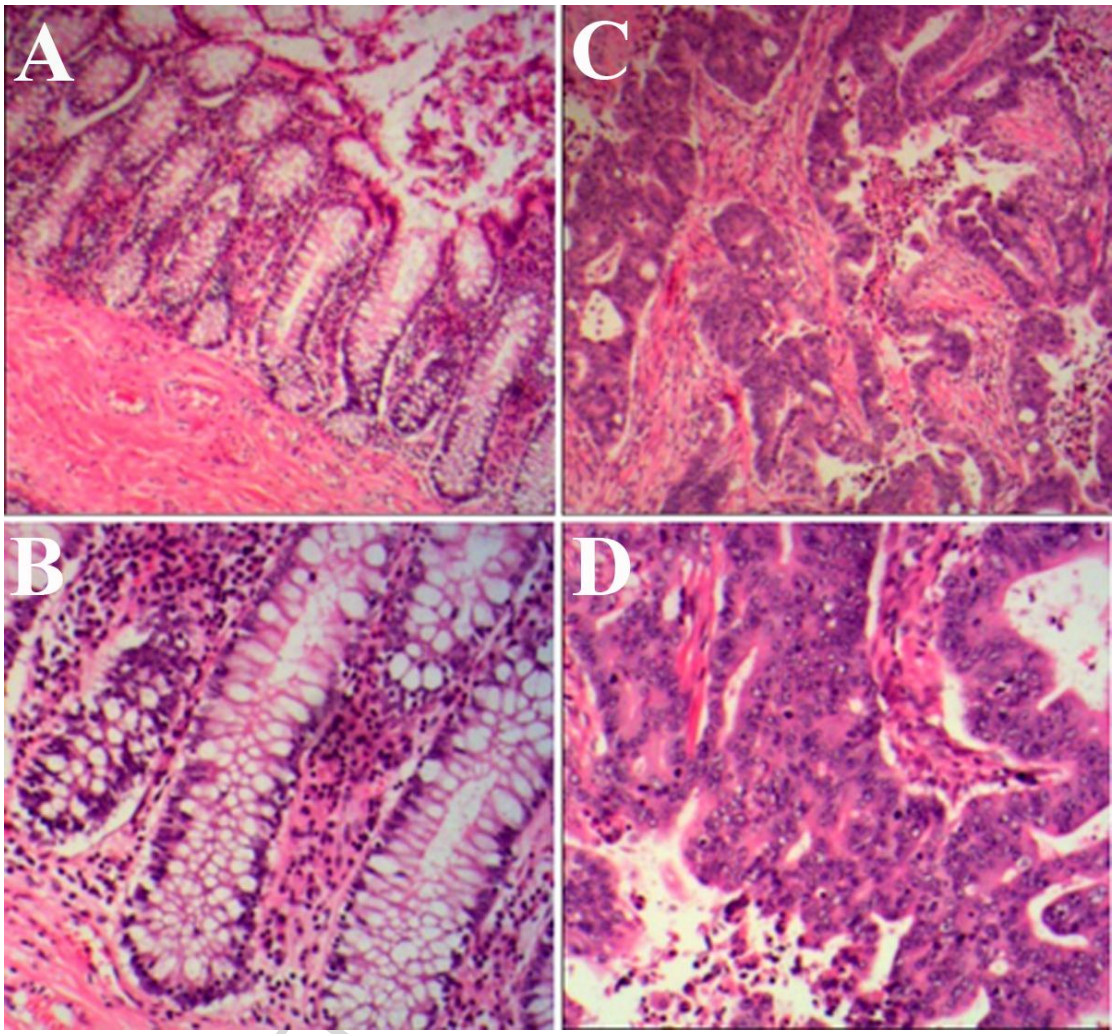

Fig. 1

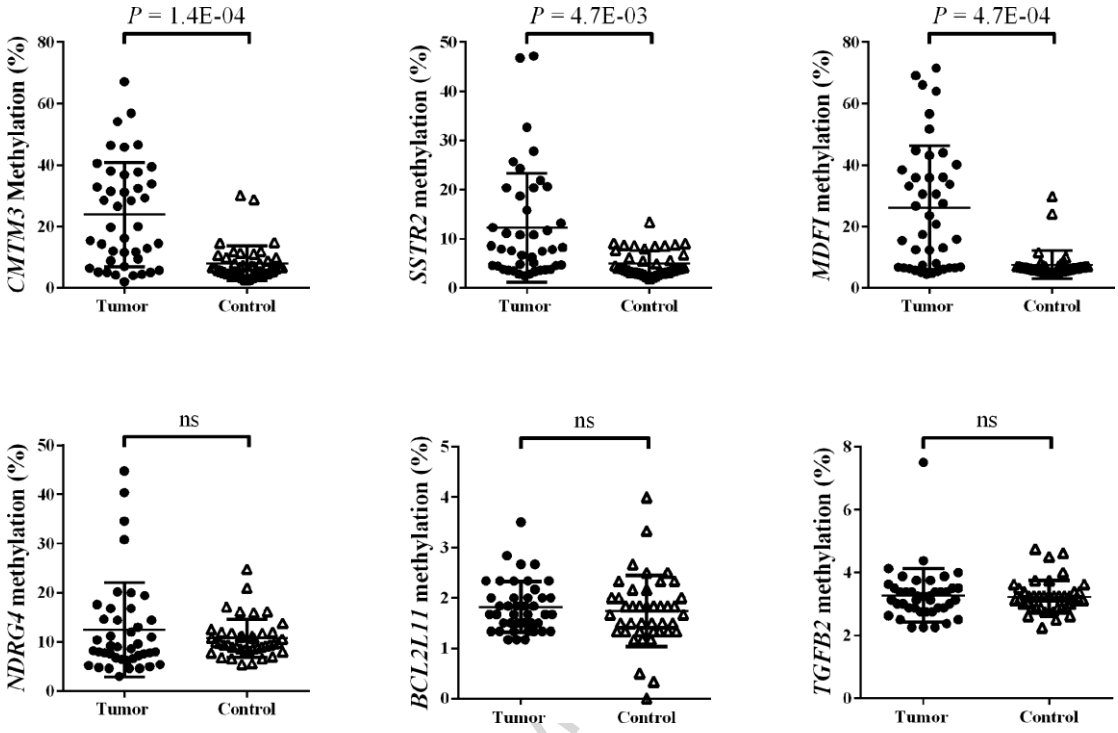

Fig. 2

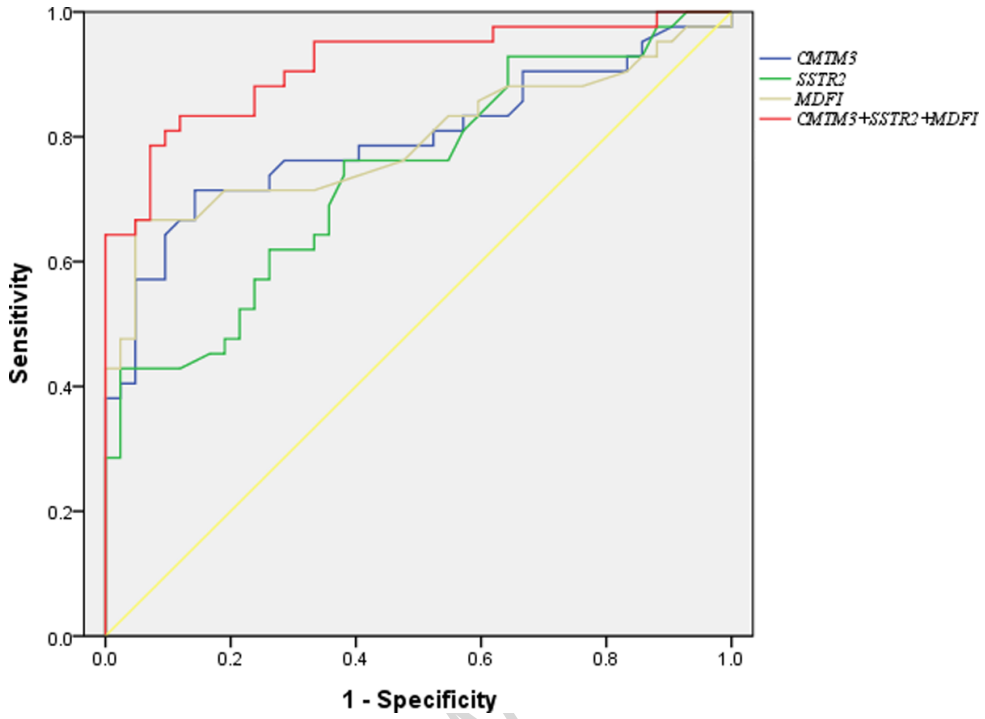

Fig. 3

Abbreviations

NSCLC: non-small cell lung cancer

PCR: Polymerase chain reaction

CEA: carcinoembryonic antigen

CA19-9: carbohydrate antigen 19-9

LEF: lymphocyte enhancer factor

ROC: The receiver operating characteristic curve

AUC: the area under the curve

CRC: colorectal cancer

BCL2L11: Bcl-2 like 11

CMTM3: CKLF-like MARVEL transmembrane domain-containing member 3

NDRG4: N-Myc downstream-regulated gene family 4

SSTR2: Somatostatin receptor 2

MDFI: MyoD family inhibitor

H&E: hematoxylin and eosin

Highlights

- The hypermethylation of *CMTM3*, *SSTR2* and *MDFI* is frequently observed in colorectal cancer.
- The AUC values of *CMTM3*, *SSTR2* and *MDFI* are 0.79, 0.74 and 0.80, respectively.
- The combination of *CMTM3*, *SSTR2* and *MDFI* produces the AUC value of 0.9 with a sensitivity of 81%.