FANCF hypomethylation is associated with colorectal cancer in Han Chinese

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ABSTRACT

Background/Aims: *Fanconi anemia complement group F (FANCF) is known to be involved in DNA repair, and the overexpression of FANCF protein leads to cell proliferation and ultimately to cancer. The purpose of this study was to assess whether FANCF methylation was associated with colorectal cancer (CRC).*

Materials and Methods: *A case-control experiment was conducted to study the association between FANCF methylation and CRC. We used quantitative methylation-specific PCR to measure the FANCF promoter methylation, and the percentage of methylation reference (PMR) to quantify the FANCF promoter methylation level. To investigate the effect of the selected FANCF fragment on gene expression regulation, we also performed a dual-luciferase reporter gene assay.*

Results: *The results indicated that FANCF methylation in CRC tumor tissues was significantly lower than that in the nontumor tissues (median PMR: 44.86% vs. 65.77%, p=0.00001). Analysis of receiver-operating characteristic curves showed that FANCF hypomethylation had a diagnostic value for CRC (area under curve [AUC]: 0.670, sensitivity: 55.8%, specificity: 71.7%, p=0.00001). The dual-luciferase reporter assay showed that the FANCF fragment upregulated gene expression (fold change: 1.93, p=0.002).*

Conclusion: *Research demonstrates for the first time that FANCF hypomethylation is significantly associated with CRC risk. FANCF hypomethylation may ultimately increase the risk of CRC by upregulating the expression of FANCF.*

Keywords: *FANCF, colorectal cancer, DNA methylation, hypomethylation, SYBR green-based qMSP assay*

INTRODUCTION

The global annual incidence of colorectal cancer (CRC) is approximately 1.3 million (1), and it is one of the most common malignancies in the world (2). The high rate of CRC has placed a significant burden on the society and families (3). The average 5-year survival rate in the early stage of CRC can be as high as 90%. However, the mid-to-late stage CRC is generally associated with cancer metastasis, which results in an average 5-year survival rate of CRC of less than 10% (4). Fecal occult blood test (FOBT) and colonoscopy are the 2 main methods widely used in the clinical screening of CRC (5). However, FOBT is susceptible to external factors, such as drugs and diets, leading to false positives, which are expensive and invasive; therefore, FOBT is not feasible for CRC screening in the population (5).

CRC is a malignant disease that is involved with multiple risk factors, including both environmental and genetic aspects (6). Epigenetics also plays a vital role in the occurrence and development of CRC (7). DNA methylation, one of the essential epigenetic modifications (8), may lead to abnormal gene expression. Silencing of tumor suppressor genes (9, 10) and abnormal activation of proto-oncogenes are considered to be one of the underlying molecular mechanisms of CRC development and progression (11, 12).

The Fanconi anemia complement group F (*FANCF*) locates on chromosome 11p15, a region enriched in cancer-associated genes (13). The Fanconi anemia (FA) protein is composed of 15 FA complementary groups of multifunctional proteins (14-16). As a molecular adaptor in the FA core complex, *FANCF* is primarily involved in the monoubiquitination of the downstream protein (FANCD2) in the FA/breast cancer susceptibility gene repair pathway (17, 18). Studies have shown that downregulation of *FANCF* expression can inhibit proliferation, migration, and invasion of breast cancer cells (19), suggesting that *FANCF* silencing may be critical in early

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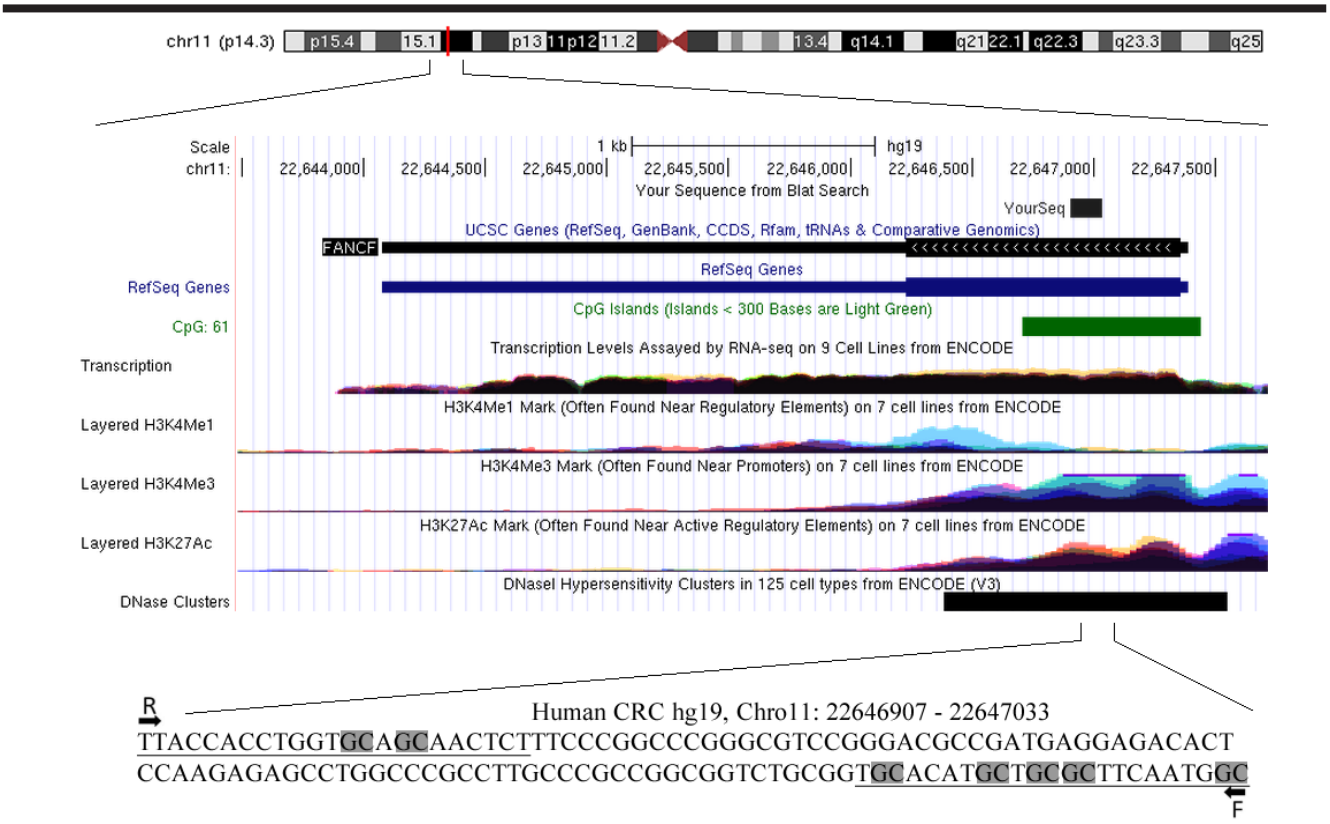


Figure 1. The characteristics of the target sequence in FANCF. The target sequence (chr11:22646907-22647033) was located on the promoter of FANCF. F and R represented forward and reverse primers, respectively.

carcinogenesis (20). Aberrant methylation of *FANCF* has been reported to occur in a variety of cancers, including ovarian tumors (17, 21), non-small-cell lung cancer (22), cervical cancer (23), and oral cancer (24).

Given the results of previous studies, we concluded that *FANCF* methylation might play a potential role in the development of CRC. There are currently no reports of *FANCF* methylation in CRC, so the goal of this study was to determine whether *FANCF* methylation was associated with the risk of CRC.

MAIN POINTS

- *The first report on the correlation between FANCF methylation level and CRC.*
- *Both FANCF hypermethylation and overexpression of FANCF protein could lead to inactivation of (FA) /BRCA signaling pathway, inducing CRC.*
- *Combined with other diagnostic techniques, FANCF hypomethylation could be used as an auxiliary biomarker for early diagnosis of CRC.*

MATERIALS AND METHODS

Tissue samples

This study involved tumor tissues and their adjacent nontumor tissues (5 cm from the tumor) from 113 patients with CRC. These patients were from Zhejiang Cancer Hospital (Zhejiang, China), Shaoxing First People's Hospital (Zhejiang, China), and the Third Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Nanjing, China). There were 74 male and 39 female patients with CRC, and their mean ages were 62 and 60 years, respectively. All the patients with CRC did not receive radiation or chemotherapy before surgical resection. The Ethics Committee of Ningbo University approved the study. All the participants signed a written informed consent form.

DNA extraction, bisulfite conversion, and quantitative methylation-specific PCR (qMSP) assay

DNA extraction of the tissue samples and subsequent bisulfite conversion steps were detailed in our previous publication (25). LightCycler® 480 (Roche Diagnostics, Mannheim, Germany) was used for the Synergy Brands

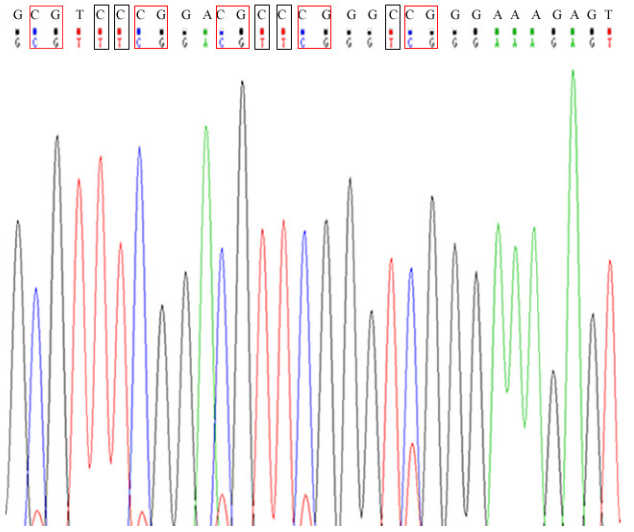


Figure 2. Sequencing validation of the product. The top line was the original gene sequence, and the next row was the DNA sequence after bisulfite conversion. The locus of CT transformation was circled by the black box, and the CG locus was circled with the red box. Sequencing results confirmed a successful bisulfite conversion.

(SYBR) green-based qMSP assay. We used β-actin (ACTB), fully methylated sperm DNA, and nuclease-free water as internal, positive, and negative controls, respectively (25). The total volume of the qMSP reaction was 10 µL, containing 0.25 µL of primer, 1 µL of transforming DNA, 4 µL of ddH_2O, and 5 µL of 2×SYBR Green Master Mix. The primer sequences of *FANCF* were 5′-GTTATTGAAGCGTAGTATGTGTA-3′ (forward primer) and 5′-TTACCACCTAATACAACAACTCT-3′ (reverse primer). The primer sequences of ACTB were 5′-TGGTGATGGAGGAGGTTTAGTAAGT-3′ (forward primer) and 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′ (reverse primer). We calculated the percentage of methylated reference (PMR) of *FANCF* by $2^{-\Delta\Delta Ct}$ quantitative detection method, where ΔΔCt=sample DNA (Ct_{FANCF}–Ct_{ACTB})–fully methylated DNA (Ct_{FANCF}–Ct_{ACTB}) (26).

Plasmid transfection and dual-luciferase reporter gene assay

Plasmid construction and its transfection into the human embryonic kidney 293T (HEK293T) cell line were carried out as previously described (27). We measured the luciferase activity using a dual-luciferase reporter assay system (Dual-Luciferase Reporter Assay Systems, Promega, Madison city, WI, USA). We cloned a fragment containing the *FANCF* methylation detection region into the pGL3-basic vector using a DNA ligation kit (Takara, Japan). The pGL3-basic and pGL3-promoter vectors

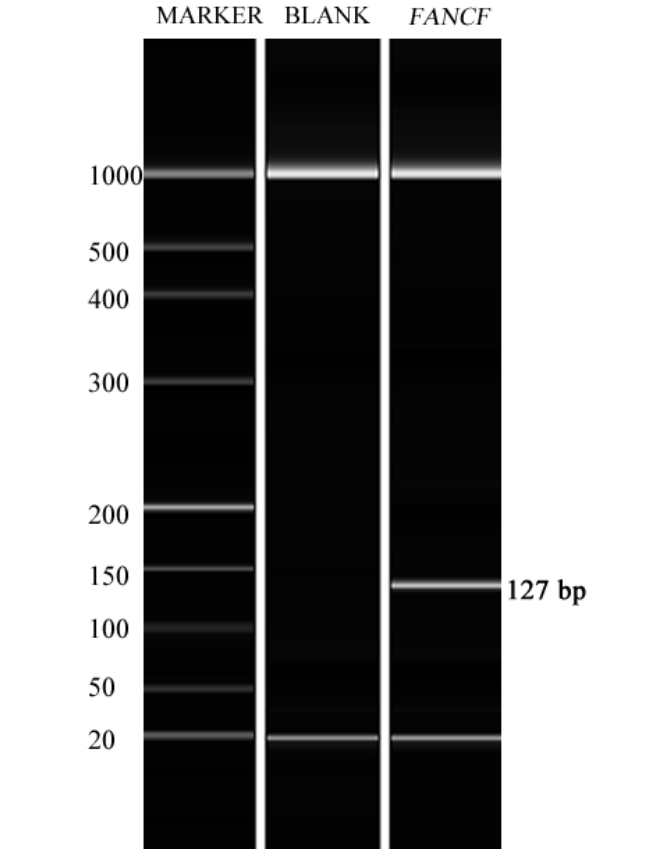


Figure 3. The results of capillary electrophoresis. The first column was the marker band; the second column was the blank; and the third one was the *FANCF* amplicon band. The electrophoresis result confirmed that the fragment length was 127 bp as expected.

(Promega, Madison city, WI, USA) were used as negative and positive controls, respectively. Luciferase activity was normalized to Renilla luciferase activity, which was determined by SpectraMax 190 instrument (Molecular Devices, Sunnyvale, USA).

Statistical Analysis

Paired sample *t*-test and nonparametric Wilcoxon test were used to compare the PMR values between the groups. Descriptive data were expressed as mean±standard deviation or median (interquartile range). The receiver-operating characteristic (ROC) curve analysis was used to compare the diagnostic performance of *FANCF* methylation for CRC. A 2-sided p value less than 0.05 was considered significant.

RESULTS

FANCF is located on chromosome 11 and contains only 1 intron-free exon (Figure 1). The detection fragment in

Table 1. Comparison of FANCF methylation between CRC tumors and paratumors.

Variables	Number	Tumor PMR (%)	Paratumor PMR (%)	p*
Total	113	44.86 (29.22, 70.44)	65.77 (44.95, 101.45)	0.00001[a]
Gender				
Male	74	44.84 (28.12, 79.06)	62.99 (42.78, 97.01)	0.002 [a]
Female	39	44.86 (29.65, 59.60)	77.29 (49.15, 107.80)	0.001[a]
Age (years)				
≥61	62	46.26 (32.93, 74.26)	64.52 (40.55, 98.59)	0.041[a]
<61	51	39.87 (21.49, 67.64)	66.01 (47.95, 105.50)	0,00003[a]
Differentiation				
Poorly	18	58.17±37.17	64.62±21.88	0.528[b]
Moderate+well	91	44.59 (29.54, 67.64)	69.85 (41.86, 107.80)	0.00006[a]
Lymph node metastasis				
Yes	59	42.39 (25.22, 65.36)	63.47 (47.91, 94.53)	0.001[a]
No	54	45.30 (32.93, 79.06)	67.93 (41.05, 120.40)	0.002[a]
Tumor location				
Colon	45	45.74 (29.78, 70.44)	61.26 (46.60, 140.10)	0.007[a]
Rectum	57	44.59 (25.11, 79.06)	70.69 (44.47, 97.73)	0.0007[a]
Colon+rectum	8	43.96±12.22	125.17±187.62	0.243[b]

*The numerical values in bold are statistically significant (p<0.05),

[a]The data did not conform to the normal distribution. Therefore, the p value was calculated by a nonparametric rank test, and it was expressed as a median (interquartile range).

[b]Since the data conform to the normal distribution, independent sample t-test was applied, and the results were described as mean±SD (standard deviation).

CRC: colorectal cancer; FANCF: Fanconi anemia complement group F; PMR: percentage of methylated reference.

this methylation experiment is 127 bp in length and is located in the *FANCF* promoter region. Sanger sequencing results showed complete bisulfite conversion (Figure 2). Capillary electropherogram results confirmed that the length of the qMSP product was as expected (Figure 3).

Our results showed that the methylation level of *FANCF* in CRC tumor tissues was significantly lower than that in the adjacent nontumor tissues (median PMR: 44.86% vs. 65.77%, p=0.00001, Table 1, Figure 4). Between the tumor and nontumor tissues, we analyzed the effects of various clinical indicators on *FANCF* methylation (Table 1). Our analysis showed that in patients with moderately and highly differentiated tumors, CRC tumor tissues had lower *FANCF* methylation levels than nontumor tissues (median PMR: 44.59% vs. 69.85%, p=0.00006), whereas there was no significant association between *FANCF* methylation and CRC risk in patients with differentiated tumors (p=0.528). For patients with CRC in whom the cancer tissues were located in the colon (*n*=45, p=0.007) or rectum (*n*=57, p=0.0007), the level of *FANCF* methylation in the tumor tissues was lower than that in the nontumor tissues; however, in patients with CRC in whom there were both colon and rectal tumors, *FANCF* methylation was not associated with CRC (*n*=8, p=0.243).

The AUC of *FANCF* hypomethylation was 0.670 (95% confidence interval [CI]: 0.599-0.740) with a sensitivity of 55.8% and a specificity of 71.7% (Figure 5a), suggesting that *FANCF* hypomethylation could be used as a biomarker for CRC. Subsequently, a subgroup analysis in patients with CRC with moderate and well-differentiated tumors also showed result similar to the abovementioned result (AUC: 0.673, 95% CI: 0.595-0.751, sensitivity:

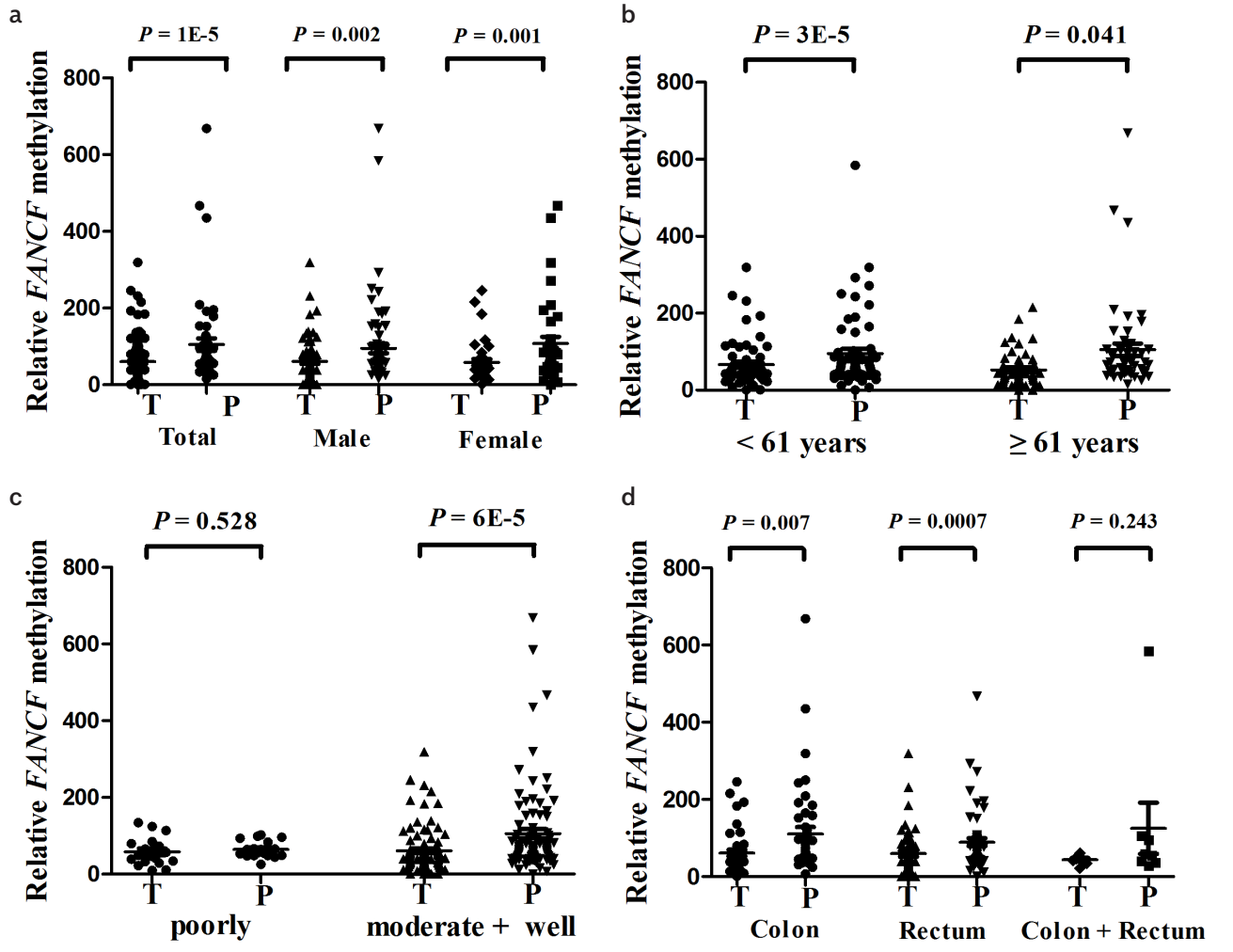


Figure 4. a-d. Comparison of *FANCF* methylation levels between different groups.
Comparison of *FANCF* methylation levels between tumor tissues and paired adjacent nontumor tissues by a) total and gender, b) age, c) differentiation, d) tumor location. T denoted tumors; P denoted adjacent nontumor tissues.
FANCF: Fanconi anemia complement group F.

59.3%, specificity: 71.4%, p=0.00006, Figure 5b). In addition, *FANCF* hypomethylation had a diagnostic value for patients with CRC with tumors located in the colon (AUC: 0.676, 95% CI: 0.565-0.786, sensitivity: 93.3%, specificity: 37.8%, p=0.007, Figure 5c) and for those with rectal tumors (AUC: 0.669, 95% CI: 0.569-0.769, sensitivity: 66.7%, specificity: 66.7%, p=0.0007, Figure 5d).

Subsequently, we used the dual-luciferase reporter system to detect whether the target fragment of *FANCF* had gene promoter activity. Our results showed that the transcriptional activity of the pGL3-*FANCF* plasmid increased significantly than the pGL3-basal vector (fold change (FC): 1.93, p=0.002, Figure 6). Further bioinformatics analysis of the TCGA database (http://www.cbioportal.org/) showed an inverse correlation between *FANCF* expression and *FANCF* methylation (r=−0.345, p=0.00006). GEO data analysis found that the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine increased the expression of *FANCF* (GEO accession number: GSE4717, FC: 1.13, p=0.00002).

DISCUSSION

Our results showed that there was significant *FANCF* hypomethylation in CRC, and the dual-luciferase reporter gene system indicated that the *FANCF* fragment could

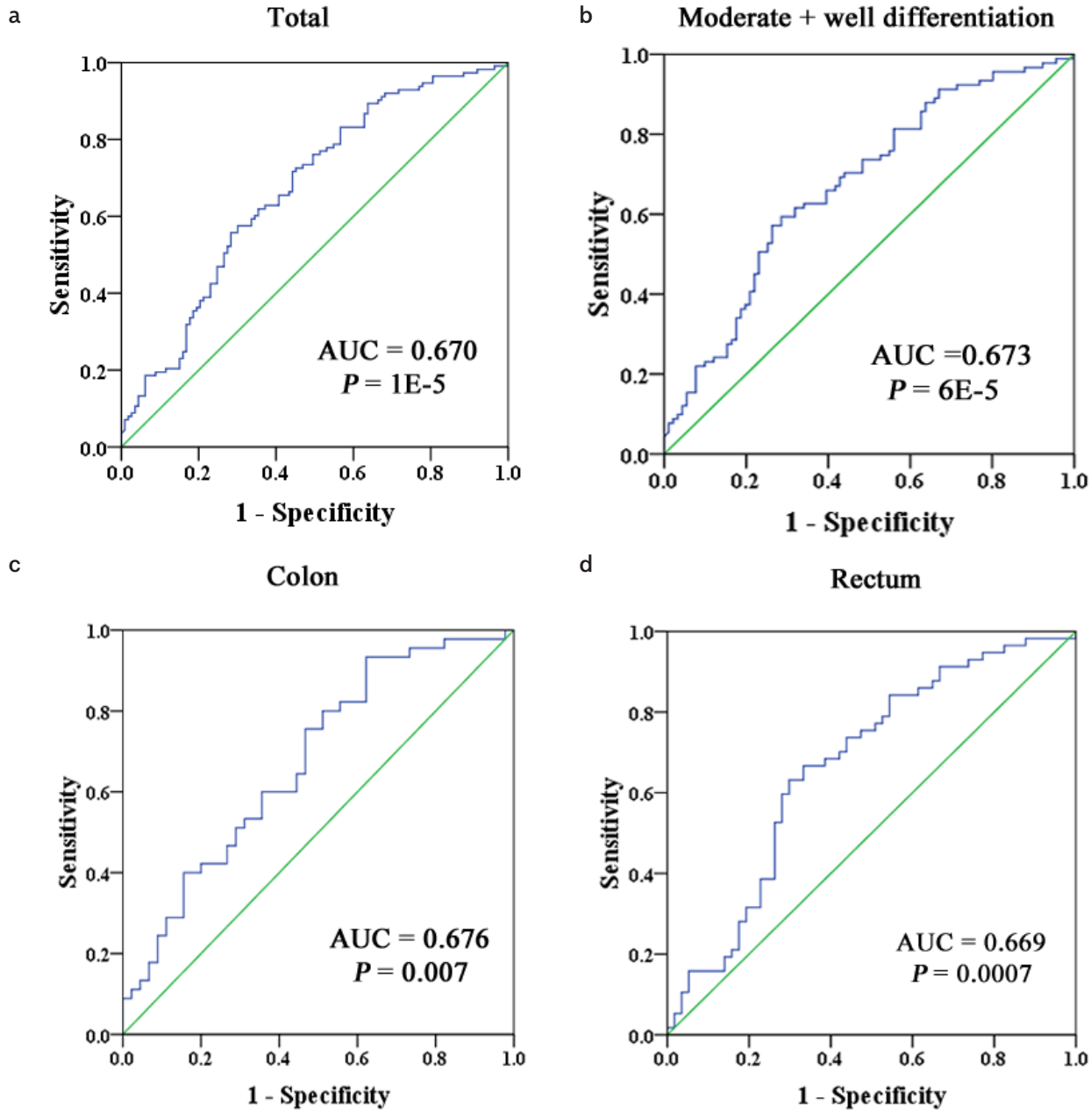


Figure 5. a-d. Receiver-operating characteristic (ROC) curve of *FANCF* methylation for its diagnostic value of CRC. The ROC curves obtained from the analysis between tumors and nontumors in a) total patients, b) patients with moderate and well-differentiated tumors, c) patients with tumors at the colon, d) patients with tumors at the rectum.
AUC: area under the curve; 95% CI: 95% confidence interval; CRC: colorectal cancer; *FANCF*: Fanconi anemia complement group F.

promote gene expression. TCGA and GEO data analysis showed that *FANCF* methylation was inversely correlated with its mRNA expression. Our results suggested that *FANCF* hypomethylation was associated with CRC risk and might be useful for early CRC screening.

FANCF is a gene in the FA/BRCA pathway, which is involved in the regulation of cell cycle, DNA damage and repair, apoptosis, gene transcription, and gene stability (22, 28). There is evidence that the FA/BRCA pathway is in equilibrium, and low expression and overexpression of

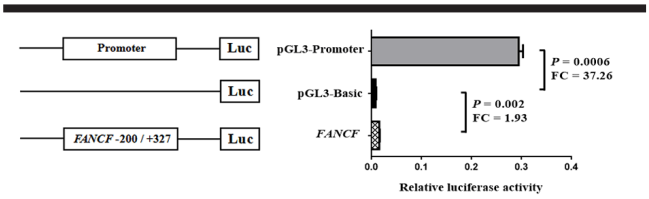


Figure 6. The dual-luciferase reporter assay in HEK-293T cell lines. The pGL3-basic vector denoted negative control, and the pGL3-promoter vector denoted positive control p values of pGL3-basic vector and pGL3-Promoter-*FANCF* vector were 0.0006 and 0.002, respectively. FC: fold change.

FANCF protein may interfere with this balance (24). The previous report showed that *FANCF* hypermethylation existed in ovarian cancer (21), non-small-cell lung cancer (22), cervical cancer (23), and oral cancer (24). However, a Japanese study on breast cancer indicated that only 4 of the 99 patients had *FANCF* methylation (29), and a Chinese research showed that none of the 102 GC patients had *FANCF* methylation (30). However, most of the studies used the MSP method, which is a qualitative methylation level measurement method with lower accuracy than the qMSP method (31). Our results showed that *FANCF* methylation levels in the CRC tumor tissues were significantly lower than the adjacent nontumor tissues, and studies have shown that demethylation of *FANCF* leads to overexpression of *FANCF* mRNA (22), resulting in resistance to the FA/BRCA pathway (22, 28). Interestingly, FANCF inhibition was found to be associated with decreased proliferation, migration, and invasion potential; thus, FANCF was likely to be a supportive, promising therapeutic target (19). In this study, TCGA data mining found an inverse correlation between *FANCF* methylation and FANCF protein expression. GEO database analysis found that *FANCF* demethylation can lead to transcriptional upregulation. We hypothesized that *FANCF* hypomethylation might lead to the development of CRC by inducing *FANCF* overexpression, thereby interfering with the FA/BRCA pathway and ultimately leading to cancer.

As a significant feature of the late stage (32), poorly differentiated tumors are more susceptible to CRC metastasis than moderately differentiated tumors, and both well-differentiated and poorly differentiated tumors positively correlate with tumor infiltration depth (33). In this study, we discovered significantly lower *FANCF* methylation in the invasive colon and rectal cancers and in moderately and highly differentiated CRC tumors but not in the poorly differentiated tumors. Our results suggest that *FANCF* hypomethylation is specific to the early stage of CRC.

Currently, FOBT (sensitivity: 33.3%, specificity: 90.9%) (34) and carcinoembryonic antigen (sensitivity: 80.0%, specificity: 70.0%) are the 2 diagnostic methods for CRC (35). Our ROC curve analysis showed that *FANCF* hypomethylation had a relatively moderate sensitivity of 55.8% and a specificity of 71.7%, suggesting that *FANCF* methylation may be a diagnostic predictor of CRC.

In summary, our results emphasize that *FANCF* hypomethylation may be associated with CRC. In future work, it is necessary to clarify the specific molecular mechanism of *FANCF* hypomethylation in the development of CRC.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Zhejiang Cancer Hospital (No: IRB-2018-28).

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - H.Y., R.P.; Design - H.Y., S.D.; Supervision - T.G., S.D.; Resource - T.G., D.W.; Materials - T.G., D.W.; Data Collection and/or Processing - R.P., J.Y.; Analysis and/or Interpretation - H.Y., S.D.; Literature Search - T.G., J.Y.; Writing - H.Y., R.P., S.D.; Critical Reviews - D.W., J.Y.

Conflict of Interest: The authors have no conflict of interest to declare.

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