

## Research Paper

## Highly specific multiplex DNA methylation detection for liquid biopsy of colorectal cancer



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## ARTICLE INFO

## Keywords:

Colorectal cancer  
Liquid biopsy  
DNA methylation  
Circulating tumor DNA  
Biomarkers

## ABSTRACT

**Background:** Circulating tumor DNA (ctDNA) has emerged as a useful biomarker for cancer detection and prognosis. In this study, we developed a strategy for developing a highly specific multiplex qPCR assay to detect methylated ctDNA in the blood of colorectal cancer (CRC) patients and investigated the potential use for the detection and prognosis of CRC.

**Methods:** Bisulfite conversion and amplicon sequencing were used to confirm potential CRC-specific DNA methylation markers. The selected DNA methylation candidates were validated by qMSP. The six best-performing markers were used to develop a new single-tube multiplex quantitative methylation-specific PCR assay (mqMSP). The mqMSP assay was applied to analyze plasma samples from 114 CRC patients, 47 patients with advanced adenoma, 45 patients with benign polyps, and 57 healthy controls. The clinical performance of the assay and associations with clinical outcomes were assessed.

**Results:** Six DNA methylation biomarkers were confirmed to be specifically hypermethylated in CRC tumor tissues. The newly developed mqMSP assay detected CRC with extremely high specificity (specificity of 98.2 %, with sensitivity of 67.5 %). The detection rate of ctDNA was significantly correlated with tumor size and clinical stage, with ctDNA methylation levels in the blood markedly increased with larger tumor size, poor differentiation, and advanced stage. Moreover, high preoperative methylated ctDNA level was associated with worse recurrence-free survival and overall survival.

**Conclusion:** We provided a strategy for identification of multiple highly-specific DNA methylation markers for designing multiplex DNA methylation assays for liquid biopsies of CRC. The newly developed assay has potential for CRC early detection, and prognosis.

**Abbreviations:** ctDNA, circulating tumor DNA; CRC, colorectal cancer; qMSP, quantitative methylation specific PCR; mqMSP, multiplex quantitative methylation specific PCR; FOBT, fecal occult blood test; FIT, fecal immunochemical test; CEA, carcinoembryonic antigen; NGS, next-generation sequencing; AA, advanced adenoma; BP, benign polyps; HC, healthy controls; RFS, recurrence free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; MRD, minimally residual disease.

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<https://doi.org/10.1016/j.cca.2024.120026>

Received 5 August 2024; Received in revised form 24 October 2024; Accepted 31 October 2024

Available online 2 November 2024

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## 1. Introduction

Colorectal cancer (CRC) is the most common malignancy of gastrointestinal tract and a leading cause of cancer mortality worldwide [1]. The 5-year survival rate of CRC patients in China is only 56.9 % [2]. Cancer stage at diagnosis is the most important survival predictor [3]. Regular screening, early detection, and early treatment of CRC can achieve effective prevention and even cure. In China, fecal occult blood test (FOBT), fecal immunochemical test (FIT), colonoscopy, and carcinoembryonic antigen (CEA) are routine clinical examinations for CRC [4,5]. As a noninvasive and low-cost test, FOBT is widely used. However, the false-positive rate is high due to interfering factors [6,7]. FIT has better sensitivity for CRC compared with FOBT, but it is still subject to the influence of other intestinal diseases [8]. Compliance for colonoscopy is low due to its invasiveness, cost, and risk of complications [9,10]. CEA is a serum biomarker widely used for monitoring of CRC recurrence, but it is not an early detection marker for CRC due to its lack of sensitivity [5,11]. The unmet medical need is to develop an accurate, easy to perform, accessible, inexpensive, and minimally invasive molecular test for CRC early diagnosis and prognosis.

Liquid biopsies using circulating tumor DNA (ctDNA) are gaining a lot of interest for cancer early screening, treatment efficacy evaluation and prognosis [12,13], as such markers may reflect tumor load and molecular characterization of tumor cells [14]. Many studies have shown that genetic and epigenetic alterations in ctDNA are promising markers for liquid biopsies [12–14], with some tests approved by government agencies [15,16].

CpG island hypermethylation in promotor regions of tumor suppressor genes is a common and early acquired epigenetic change in cancer pathogenesis [17,18]. Thus, detection of aberrant DNA methylation in body fluids has potential for diagnosing CRC in its early stages and monitoring disease progression and treatment response. DNA methylation markers for early detection of CRC have been studied extensively [19,20]. However, lack of systematic approaches in identifying marker panels, limited sensitivity and/or limited specificity have hindered its clinical applications. Tests with a single marker, such as SEPT9 methylation, have insufficient sensitivity for early-stage CRC [21]. Combinations of multiple markers can increase the sensitivity for cancer detection, but they may suffer from decreased specificity [22,23].

Sensitivity and specificity are the most important indicators reflecting the accuracy of ctDNA tests. Currently, different ctDNA testing products may focus on different strategies to apply to specific clinical scenarios. A highly sensitive diagnostic test is important where the test is used to identify a serious but treatable disease; and a highly specific test avoids further subjection of the patient to unnecessary follow-up medical procedures. For example, most liquid biopsy platforms in advanced stage of development, including the current market leaders Galleri (GRAIL) and CancerSEEK (Thrive), have calibrated their tests to achieve a very high specificity while sacrificing sensitivity, in order to avoid false positives that may lead to excessive testing, unnecessary panic among healthy subjects, as well as additional diagnosis, treatment, and expenses in the later stage [24–26]. For tests such as Delfi, which is lung-cancer specific and offered in a population eligible for lung cancer screening, using a lower specificity ensures that as many people with cancer are detected even if there are false-positives, since a positive test leads to standard of care low-dose computed tomography (LDCT) [27].

We have previously developed a single-tube multiplex quantitative methylation-specific PCR (mqMSP) assay for ctDNA analysis for CRC detection and early prediction of CRC recurrence. While the mqMSP assay showed significant improvement in precancerous and early stage CRC detection over the single marker SEPT9 approach, its overall specificity may need further improvement [28]. In some clinical scenarios, it is more appropriate to choose a more specific assay. To further optimize this method, we developed a strategy to further prioritize DNA methylation markers for multiplex detection in plasma, and hypothesized that the diagnostic accuracy rate can be improved with the new

assay. These improvements included the introduction of new biomarkers, more simplified experimental procedures, easier interpretation of results, and lower costs. We further evaluated the clinical usefulness of this test for CRC detection and prognosis.

## 2. Materials and methods

### 2.1. Patients and samples

This study was approved by the medical ethics committee of the First Affiliated Hospital of Wenzhou Medical University. All patients were confirmed by colonoscopy and pathologic examination when necessary. Advanced adenoma was defined as adenoma with either of the following characteristics:  $\geq 10$  mm in size or high-grade dysplasia. Healthy controls were selected based on the absence of hyperplastic and adenomatous polyps by colonoscopy (but allowing benign conditions, such as diverticular disease and hemorrhoids). Patients' CEA and CA19-9 results were obtained from the routine preoperative examination after admission, which were measured by Cobase automatic electrochemical luminescence instrument according to the manufacturers' instructions.

### 2.2. DNA extraction, bisulfite conversion and methylation analysis

Genomic DNA was extracted from tissue samples using the QIAamp DNA Mini Kit (Qiagen). Buffy coat DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen). For plasma samples, 2–5 mL plasma was extracted using the Apostle MiniMax High-Efficiency cfDNA Isolation Kit (Apostle). For bisulfite conversion, 1  $\mu$ g of tissue DNA or 5–20 ng of plasma DNA were converted with the Zymo EZ DNA Methylation-Gold Kit (Zymo). All the kits were used according to the manufacturers' instructions. Purified DNA obtained from the above steps was tested by bisulfite cloning and sequencing or methylation-specific real-time qPCR. All primers and probes were synthesized by GenScript Biotechnology, and sequences are listed in Supplement, Table S1.

For bisulfite cloning and sequencing, the target regions were amplified, and the PCR products were TA-cloned into pGEM-T Easy vector (Promega) for cloning and sequencing. For methylation-specific real-time qPCR, target methylated marker (labeled with FAM fluorescence) and an internal control (ACTB, labeled with VIC fluorescence) can be detected simultaneously in the same multiplex qPCR reaction. The reactions were performed in a 25- $\mu$ L final volume system with 12.5  $\mu$ L of 2 $\times$ KAPA PROBE FAST qPCR Master Mix (KAPA), 0.25  $\mu$ M of each target primer, 0.1  $\mu$ M of each target probe, 0.06  $\mu$ M of each ACTB primer, 0.05  $\mu$ M of ACTB probe, nuclease-free water and bisulfite-converted DNA. PCR cycling conditions on an ABI 7500 Instrument (Applied Biosystems) were as follows: heat activation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 3 s and 60 °C for 30 s, and then 72 °C for 30 s.

### 2.3. qPCR products amplicon sequencing

Amplicon sequencing was performed on the cfDNA qPCR products from our previous research [28], including samples from 28 CRC and 9 healthy controls. qPCR product purification was performed with Oligo Clean & Concentrator kit (Zymo). Library preparation was performed using the KAPA Library Preparation Kits (KAPA), no library PCR was performed. Libraries were quantified using the Agilent DNA High Sensitivity Kit on the 2100 BioAnalyzer instrument. Then, pair-end 2 $\times$ 150 bp sequencing of the samples was performed on the HiSeq Xten NGS platform (Illumina). Sequencing data analysis was performed with Burrows-Wheeler Transform-based mapping algorithms.

### 2.4. ctDNA methylation analysis

A single PCR reaction was performed with purified bisulfite-

converted DNA from each plasma sample. Multiple target markers (labeled with FAM fluorescence) and an ACTB control (labeled with VIC fluorescence) would be detected simultaneously in the same multiplex methylation-specific real-time qPCR reaction as above described. The FAM signal was used to quantify the overall methylation signal for the six markers in the mqMSP assay, with a lower FAM Cq value representing greater DNA methylation. Positive and negative control reactions were performed with each batch of plasma samples as quality controls. Plasma DNA samples with inadequate DNA (<5 ng) were excluded. A DNA sample was considered as positive when the FAM Cq value was less than 45.

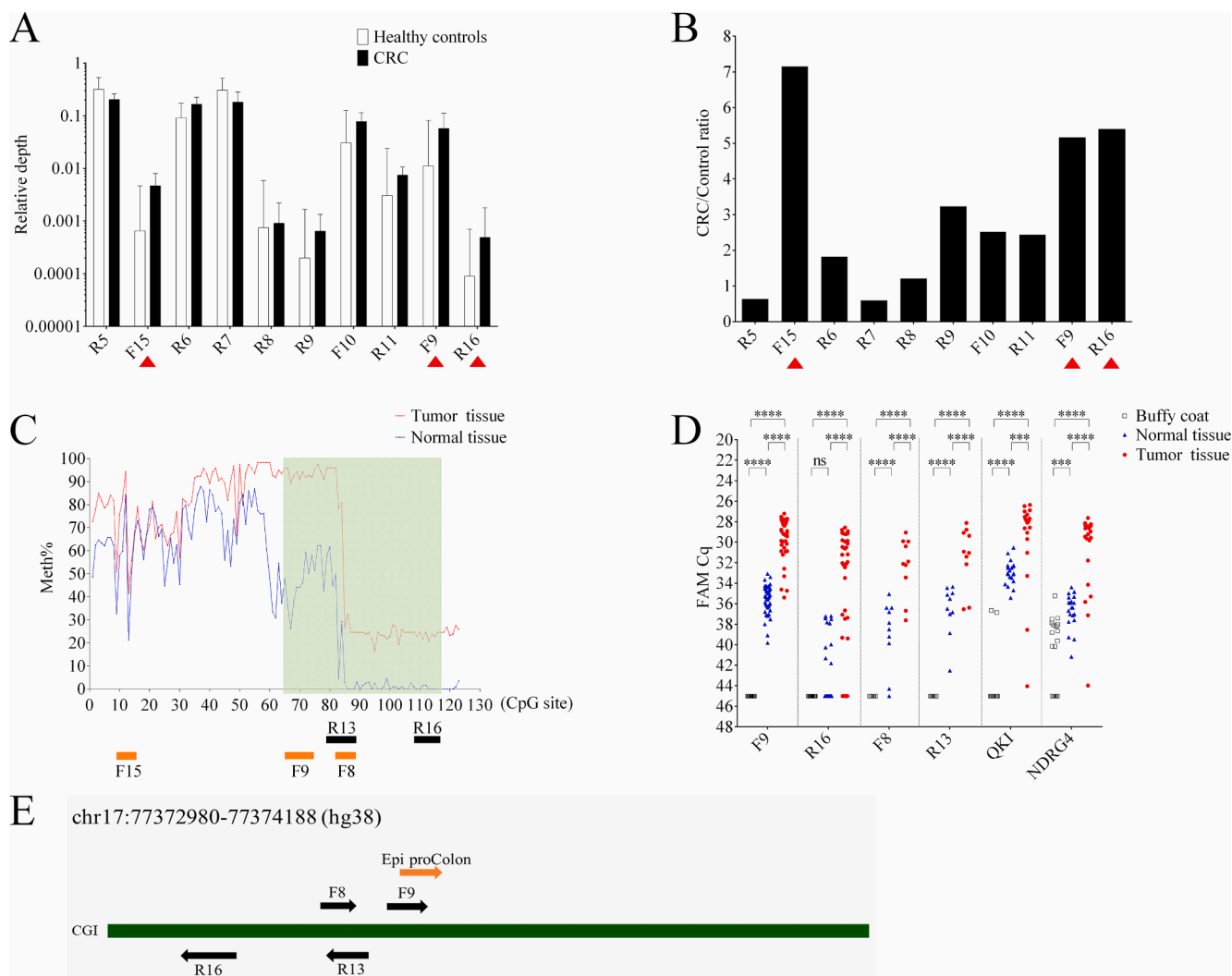
2.5. Performance evaluation of the mqMSP assay

HCT-15 cell line DNA (100 % methylated) was mixed with buffy coat DNA (not methylated for the markers) at 1 %, 0.5 % and 0 % for

performance evaluation of the mqMSP assay. The DNA mixtures were subject to bisulfite conversion and mqMSP analyses, with the total DNA amount fixed at 30 ng per reaction. DNA samples with 1 %, 0.5 %, and 0 % methylation were used to evaluate the reproducibility of the mqMSP assay. Each methylation level was tested with 10 repeats and the coefficient of variation (CV = standard deviation/mean × 100 %) was calculated. Additionally, 1 % methylated DNA were used to compare the 6-marker mqMSP assay with each individual qMSP assay.

2.6. Statistical analysis

Statistical analysis was performed using software SPSS Version 20.0 and GraphPad Prism Version 6.0. Clinical variables were described using mean±standard deviation or median (interquartile range [IQR]) as appropriate to distribution. The Wilcoxon rank-sum test and Chi-square test were used to assess the statistical significance between groups. One-



**Fig. 1.** A strategy to rigorously select candidate DNA methylation markers for liquid biopsies. (A) Amplicon sequencing results for the previously published 10-marker panel. For each marker (X-axis), the height of the histogram bar indicates signal level (Y-axis) by calculating the relative depth, where relative depth was calculated by dividing the depth of each amplicon by the sum of the total depth of 10 amplicons in each sample. (B) Signal-to-noise ratios as defined as amplicon signal in colorectal cancer samples to amplicon signal in healthy control samples, where the amplicon signal was calculated as the geometric mean of depth of each amplicon in either the colorectal cancer samples or healthy control samples. (C) Bisulfite cloning and sequencing for paired five CRC tumor and normal tissues. X-axis indicates the positions of the CpG sites within the PCR amplicons. Methylation level for each CpG was determined by calculating the average values for either the tumor or the normal tissue samples. (D) Samples from 20 buffy coat samples, 40 pairs of CRC and normal tissues were analyzed for the selected markers. Methylation level for each biomarker was calculated based on the FAM Cq value where a smaller Cq value represents higher DNA methylation level for the biomarker. \*\*\*\*P < 0.0001; \*\*\*P < 0.001; \*\*P < 0.01; \*P < 0.05; ns, not significantly different. P values refer to Fisher's least significant difference test. (E) Chromosomal locations of the 4 selected markers in the SEPT9 gene. The 4 subregions are shown with black arrows; forward arrows are for the qMSP assays designed for the forward strand, and reverse arrows are for the assays designed for the reverse strand. The Epi proColon assay is shown in an orange arrow.

way ANOVA was used to compare three or more independent populations. Patient follow-up data were collected between January 2016, and May 2023. The maximum value of the Youden index was used to define the cutoff value of ctDNA risk group. Kaplan-Meier (K-M) survival curves with log-rank test was used to assess the prognostic difference of high-risk versus low-risk group. Univariate and multivariate COX risk regression analysis are used to select and determine variables that have independent relationships with progression and OS. All statistical tests were two-sided and a p-value of <0.05 determined statistical significance.

### 3. Results

#### 3.1. Identification of CRC-specific DNA methylation biomarkers and optimization of the new mqMSP assay

We reasoned that combining multiple markers with each marker being highly specific for cancer may improve sensitivity without sacrificing specificity. Our previously published mqMSP assay achieved a high sensitivity, while the specificity may be suboptimal in certain clinical settings [28]. We suspected that some of the 10 markers in the previous panel may be less specific.

To investigate this possibility, we performed NGS sequencing for the amplification products using the published mqMSP assay using plasma DNA samples from CRC patients and healthy controls (Fig. 1A, B). We identified three markers (R16, F9 and F15) with the highest signal-to-noise ratio when comparing CRC patients and normal controls (Fig. 1A, B). Other markers were deemed as suboptimal for specificity based on the background signals in the healthy controls. In addition, we performed bisulfite cloning and sequencing for these 10 markers using tumor and normal tissue DNA samples. Marker F15 was excluded due to its methylation level in the normal tissue, while markers F8 and R13 were added to the new panel (Fig. 1C). The 4 markers we chose were located in the 2-kb CpG-rich region of the SEPT9 gene promoter (Fig. 1E). We further added two markers (QKI and NDRG4) based on literatures search and our in-house data [29]. As a result, we have a new panel of six DNA methylation markers (R16, F9, F8, R13, QKI and NDRG4).

These six markers were then subject to validation by qMSP analysis with DNA from 20 buffy coat samples, and 40 pairs of CRC and normal tissues. The result showed that the six markers were specifically hypermethylated in CRC tumor tissues as compared to paired normal tissues (Fig. 1D). In addition, all six markers had extremely low background signal in buffy coat DNA (Fig. 1D).

We then proceeded to develop a mqMSP assay using probes labeled with FAM for all six markers. Bisulfite converted DNA were quantified by this new mqMSP assay in a single PCR reaction where FAM signal was used directly to quantify the overall methylation signal for the six markers.

We evaluated the reproducibility of the mqMSP assay (Supplement Table S2 and Figure S1). The CV of the experiment ranged from 0.57 % to 0.70 % (FAM Cq), 0.53 % to 0.83 % (VIC Cq), indicating that the mqMSP assay has good reproducibility. We also compared the 6-marker mqMSP assay with each of the six singlet qMSP assays (Supplement Table S3 and Figure S2). The mqMSP assay produced a FAM Cq value which was 1.37, 3.10, 2.90, 2.58, 2.58 and 2.63 lower than the individual F9, R16, F8, R13, NDRG4 and QKI assays, respectively, suggesting that the multiplex assay is analytically more sensitive than the individual assays.

#### 3.2. Evaluation of the new mqMSP assay for analyzing plasma DNA samples

The 6-marker panel was then evaluated with an independent cohort of plasma samples using the mqMSP assay. We recruited 114 CRC patients, 47 patients with advanced adenoma (AA), 45 patients with

benign polyps (BP), and 57 healthy controls (HC) (Table 1). The detection rates were 67.5 %, 6.4 %, 11.1 %, 1.6 % for CRC, AA, BP and HC, respectively, with methylation signals of ctDNA significantly higher in the CRC patients than in other groups (Fig. 2A). The detection rates for stage I, II, III, and IV patients were 41.9 % (13/31), 75.0 % (21/28), 67.7 % (21/31), and 91.7 % (22/24), respectively, with methylation signals in stages IV patients significantly higher than patients at stages I-III (Fig. 2B).

Interestingly, the new panel achieved our primary goal of improving detection specificity based on our marker selection. The specificity values were 94.0 %, 94.1 % and 98.2 % for non-CRC (AA, BP and HC), non-CRC/AA (BP and HC), and HC, respectively. The specificity for HC in the new mqMSP was substantially improved over our previous 10-marker assay (98.2 % vs. 85.9 %), demonstrating the validity for our new strategy for selecting highly specific DNA methylation markers for liquid biopsies.

The new marker panel showed some decrease in sensitivity, particularly for early-stage cancer. We tested whether combining ctDNA with CEA and CA19-9 may improve sensitivity. Our result indicated that a combination of ctDNA, CEA and CA19-9 improved diagnostic sensitivity of CRC from 67.3 % (ctDNA alone), 33.7 % (CEA alone), 22.1 % (CA19-9 alone) to 73.1 % (Supplement, Table S4).

#### 3.3. Correlations between ctDNA methylation level and clinicopathological variables

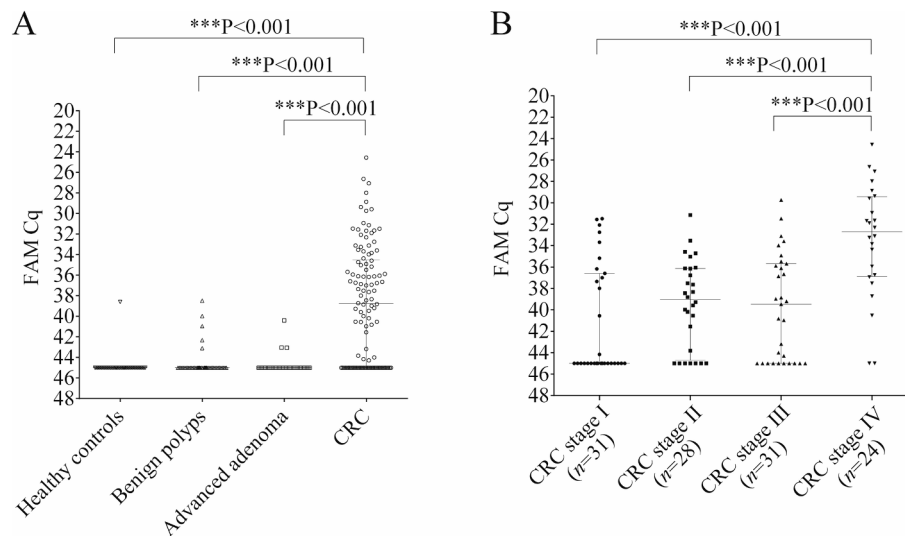
The correlations between ctDNA detection rate and the clinicopathological variables of CRC were summarized in Table 2. Tumor size, and stage, but not age, gender, tumor location, or differentiation were correlated significantly with ctDNA detection rate. The detection rates were 57.3 % and 84.8 %, respectively for tumors of <5 cm and ≥5 cm in size (P = 0.006). And the ctDNA methylation level was significantly higher for the patients with tumors ≥5 cm in size than those with tumors <5 cm in size (P = 0.0007). The detection rates were 50.0 %, 70.3 % and 80 % for tumors with high, moderate and low differentiation, respectively (P = 0.08). There was a positive correlation between ctDNA methylation level and tumor size categorized by stage or degree of differentiation (Supplement, Figure S3).

#### 3.4. ctDNA methylation in plasma for survival prediction

We further investigated whether detection of preoperative methylated ctDNA may predict recurrence free survival (RFS) or overall

**Table 1**  
Demographic characteristics of subjects tested by the new mqMSP and the positive detection rate.

Group	NO.	Male	Female	Median age (range)	Positive cases	Positive detection rate (%)
CRC						
Total	114	74	40	64 ( 45-75 )	77	67.5
Stage I	31	17	14	64 ( 53-75 )	13	41.9
Stage II	28	20	8	69 ( 45-75 )	21	75.0
Stage III	31	21	10	61 ( 47-75 )	21	67.7
Stage IV	24	16	8	62 ( 45-72 )	22	91.7
Advanced adenoma	47	36	11	58 ( 46-75 )	3	6.4
Benign Polyps	45	31	14	59 ( 49-75 )	5	11.1
Healthy controls	57	21	36	54 ( 46-74 )	1	1.8
Overall	263	162	101			



**Fig. 2. Quantification of plasma ctDNA by the new mqMSP assay.** (A) Methylation levels were represented by the FAM Cq values quantified by the new 6-marker mqMSP assay in CRC, advanced adenoma, benign polyps, and healthy controls. (B) Methylation levels of plasma DNA in different stages of CRC. Lines represent median with interquartile range. P values refer to Wilcoxon rank sum test.

**Table 2**  
Associations of clinicopathological data with ctDNA in CRC plasma samples.

	No.	Positive cases	Negative cases	Positive Detection Rate (%)	$\chi^2$	p-Value <sup>a</sup>	ctDNA methylation level <sup>b</sup>	p-Value
Age					0.951	0.864		0.804 <sup>c</sup>
<50	7	5	2	71.4			35.47 (34.34–42.91)	
50–59	24	18	6	75.0			38.52 (33.11–43.63)	
60–69	53	34	19	64.2			39.27 (35.17–45.00)	
≥70	30	20	10	66.7			37.32 (35.18–45.00)	
Gender					1.600	0.206		0.587 <sup>d</sup>
Male	74	53	21	71.6			38.76 (34.62–45.00)	
female	40	24	16	60.0			38.48 (34.78–45.00)	
Primary lesion site					0.088	0.766		0.724 <sup>d</sup>
proximal	8	5	3	62.5			36.24 (34.35–45.00)	
distal	105	71	34	67.6			38.85 (34.72–45.00)	
Tumor size					7.703	0.006		0.0007 <sup>d</sup>
<5 cm	75	43	32	57.3			40.96 (36.16–45.00)	
≥5 cm	33	28	5	84.8			36.74 (32.07–39.27)	
Tumor differentiation					5.055	0.080		0.017 <sup>c</sup>
high	28	14	14	50.0			44.58 (37.94–45.00)	
Moderate	64	45	19	70.3			38.65 (34.86–45.00)	
low	15	12	3	80.0			35.88 (33.56–38.78)	
Stage					16.355	0.001		< 0.0001 <sup>c</sup>
I	31	13	18	41.9			45.00 (36.80–45.00)	
II	28	21	7	75.0			39.04 (36.14–44.11)	
III	31	21	10	67.7			39.46 (35.78–45.00)	
IV	24	22	2	91.7			32.71 (29.53–36.79)	

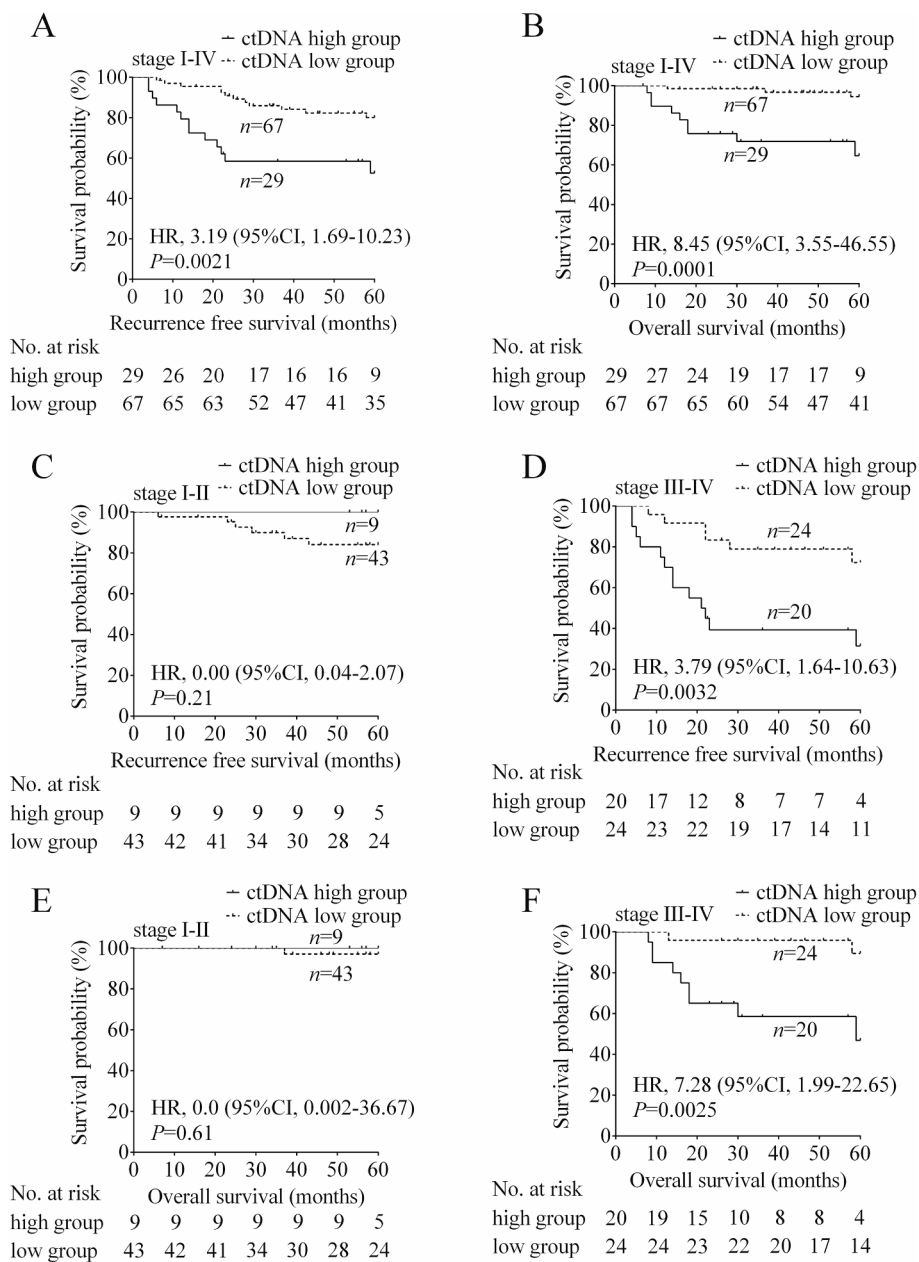
Notes: a.  $\chi^2$  test. b. Data (FAM Cq) are presented as the median (interquartile range). c. Kruskal-Wallis test. d. Wilcoxon signed rank test. \*The total number of CRC patients in some indicators is fewer than 114 due to lack of full information in several patients.

survival (OS). In survival analysis, 96 CRC patients with survival data were included, with the cutoff Cq value for mqMSP determined as 36.08 by Youden's index. CRC patients with high preoperative ctDNA methylation (FAM Cq < 36.08) at diagnosis showed significantly worse RFS and OS than those with low ctDNA methylation (FAM Cq ≥ 36.08) (p = 0.0021, HR: 3.19, 95 % CI: 1.69–10.23; p = 0.0001, HR: 8.45, 95 % CI: 3.55–46.55) (Fig. 3A, B), suggesting methylated ctDNA in the plasma has prognostic value in CRC patients. The difference is particularly significant among patients in stages III-IV (p = 0.0032, HR: 3.79, 95 % CI: 1.64–10.63; p = 0.0025, HR: 7.28, 95 % CI: 1.99–22.65) (Fig. 3D, F), but not significant in stages I-II (Fig. 3C, E).

To verify these results, we also evaluated the survival of CRC patients that in our previous study by using this new assay's algorithm [28]. 135 CRC patients with survival data in the previous dataset were included (Supplement, Table S5), with the same Cq cutoff value (FAM Cq =

36.08). CRC patients with high preoperative ctDNA methylation (FAM Cq < 36.08) at diagnosis showed significantly worse RFS and OS than those with low ctDNA methylation (FAM Cq ≥ 36.08) (p = 0.015, HR: 2.23, 95 % CI: 1.17–4.13; p = 0.0093, HR = 2.98, 95 % CI: 1.29–6.02, respectively) (Fig. 4A, B). The difference in RFS and OS were also significant among patients in stages III-IV (p = 0.0115, HR: 3.04, 95 % CI: 1.27–6.10; p = 0.0098, HR: 3.40, 95 % CI: 1.32–7.02) (Fig. 4D, F), but not significant in stages I-II (Fig. 4C, E), which is consistent with the new assay results.

The COX regression model was used to analyze clinicopathological parameters that may be used to predict prognosis. Univariate Cox regression analysis showed that TNM classification, CEA, CA199 and ctDNA were significantly associated with RFS of CRC patients (P < 0.05) (Table 3). Subsequent multivariate COX regression model showed that TNM classification and ctDNA methylation level were independent risk



**Fig. 3. Kaplan–Meier curves of RFS and OS for patients of the low- and high- ctDNA groups with new mqMSP assay.** Kaplan–Meier curves of RFS (A) and OS (B) for all patients. Kaplan–Meier curves of RFS for patients with stage I-II (C) and stage III-IV (D). Kaplan–Meier curves of OS for patients with stage I-II (E) and stage III-IV (F).

factors for RFS ( $P < 0.05$ ) (Table 3).

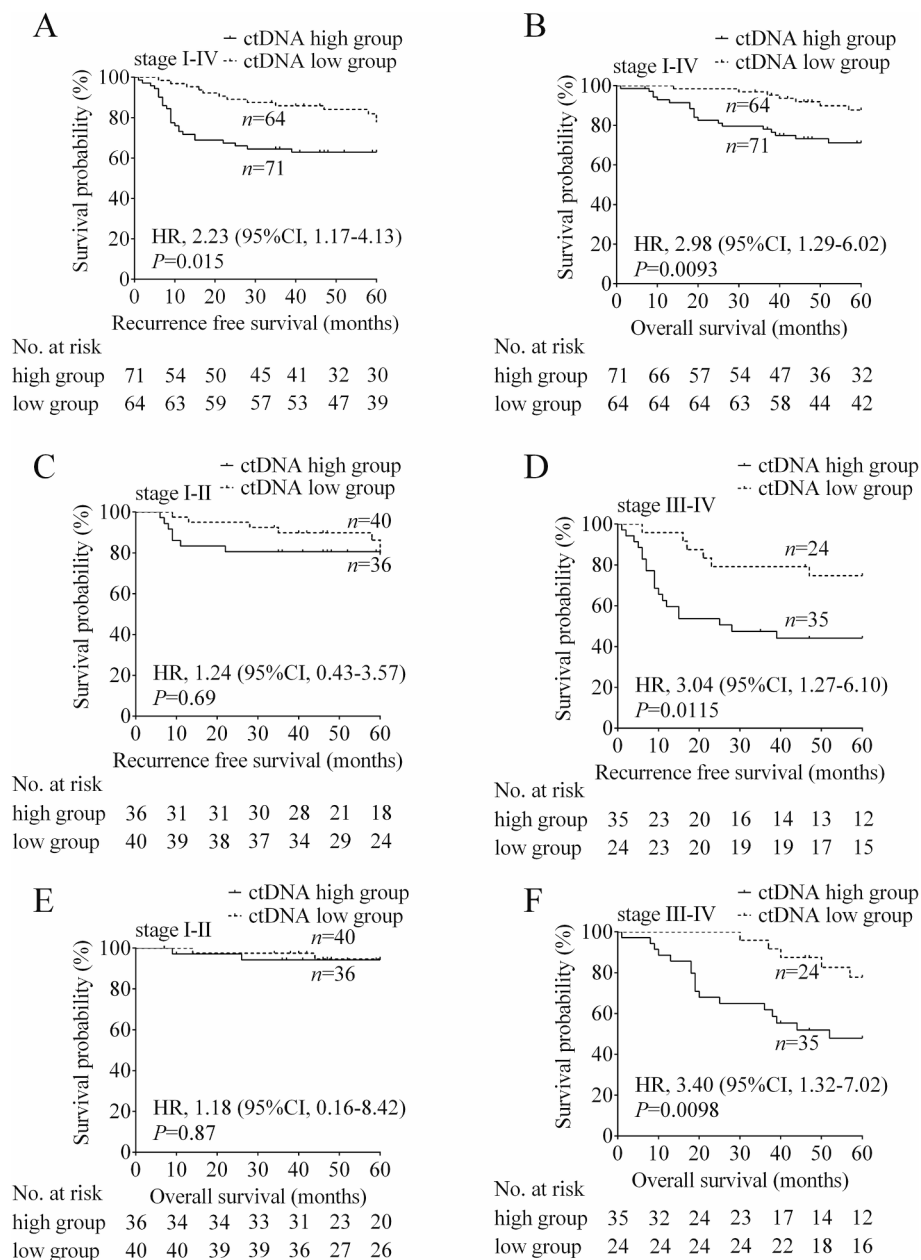
Additionally, TNM classification, CEA, CA199 and ctDNA were significantly associated with OS in univariate Cox regression analysis ( $P < 0.05$ ) (Table 4). Further multivariate Cox regression analysis indicated that CA199 and ctDNA level were significant prognostic factor for OS ( $P < 0.05$ ) (Table 4).

We also conducted the same analysis on the cohort in previous study, which showed that TNM classification, CEA, CA199 and ctDNA were significantly associated with RFS and OS in univariate Cox regression analysis ( $P < 0.05$ ) (Supplement, Table S6 and S7). Further multivariate Cox regression analysis indicated that TNM classification and CA199 were significant prognostic factor for RFS ( $P < 0.05$ ) (Supplement, Table S6), and TNM classification, CA199 and ctDNA were significant prognostic factor for OS ( $P < 0.05$ ) (Supplement, Table S7).

#### 4. Discussion

Early detection is key to increase probability for curative intervention and thus improve CRC prognosis and survival. However, there are several limitations for current strategies for CRC detection, such as high cost, invasiveness, and/or insufficient sensitivity and specificity [30]. The findings that ctDNA can be applied in cancer diagnosis, prognosis, surveillance, and treatment guidance have provided a new avenue. In this regard, circulating DNA methylation biomarkers have great potential for cancer screening, prognosis and surveillance [20,31].

In our previous study, we have demonstrated a cost-effective and easily implementable single-tube qPCR method for quantifying a panel of 10 ctDNA methylation markers for predicting CRC recurrence after curative surgery [28]. However, the previous assay, as well as many other published studies using DNA methylation markers, may need improvement in specificity. We developed a stepwise strategy to



**Fig. 4.** Kaplan–Meier curves of RFS and OS for patients of the low- and high- ctDNA groups with previously published mqMSP assay. Kaplan–Meier curves of RFS (A) and OS (B) for all patients. Kaplan–Meier curves of RFS for patients with stage I-II (C) and stage III-IV (D). Kaplan–Meier curves of OS for patients with stage I-II (E) and stage III-IV (F).

evaluate and select highly CRC-specific DNA methylation markers. Efforts were made to ensure that the selected DNA methylation markers were specifically hypermethylated in CRC compared with normal cells of hematopoietic origin. The new assay targeting six methylated biomarkers achieved 98.2 % specificity (at sensitivity of 67.5 %), which compared favorably with other similar approaches [32]. In addition, the new assay may be superior to the mSEPT9 assay (sensitivity 48.2 % at 91.5 % specificity) [21].

The much-improved specificity of the new mqMSP assay may be useful in few settings. First, in the screening setting, current stool-based assays such as FOBT and FIT may have high false positive rates. In China, the high positive rate by stool-based assays contributed to the low conversion rate (defined as the percentage of screening-positive patients opting for colonoscopy), thus effectively diminishing the benefit of screening. In this regard, the new mqMSP assay may be used in combination with the stool-based assays to improve the positive predictive

values and ultimately improving conversion rate for colonoscopy. Another potential clinical utility is MRD detection. Due to the extremely small amount of residual lesions or residual tumor cells, it is difficult to detect them through traditional imaging or other experimental methods, thus requiring more sensitive detection techniques. The current method based on MRD ctDNA analysis is mainly next-generation sequencing targeting somatic mutations. However, due to the inter-individual differences in mutation profiles, significant efforts are required for designing target panels. Our method based on methylation markers can cover most patients due to relatively low inter-individual variabilities in the chosen methylation markers.

With the improved mqMSP assay, we also found that elevated ctDNA methylation in the pre-operative blood is an independent risk factor for RFS and OS, particularly in stage III and IV patients, consistent with previously studies [33–35], suggesting that the assay may potentially be utilized for prognosis and patient management.

**Table 3**

Univariate and multivariate Cox regression analyses of variables predicting recurrence-free survival time of CRC patients (new assay).

Variables	Univariate analysis		Multivariate analysis	
	Hazard Ratio(95 % CI)	p-Value	Hazard Ratio(95 % CI)	p-Value
<b>Age</b>				
<50	Ref			
50–59	0.679 (0.079–5.815)	0.724		
60–69	1.432 (0.414–4.951)	0.570		
≥70	1.710 (0.615–4.750)	0.304		
<b>Gender</b>				
female	Ref			
male	1.815 (0.724–4.548)	0.203		
<b>Primary lesion site</b>				
distal	Ref			
proximal	0.667 (0.090–4.932)	0.692		
<b>Tumor size</b>				
<5 cm	Ref			
≥5 cm	0.868 (0.346–2.174)	0.762		
<b>Tumor differentiation</b>				
low	Ref			
Moderate	0.642 (0.133–3.090)	0.580		
high	0.947 (0.389–2.302)	0.904		
<b>TNM classification</b>				
I	Ref			
II	0.120 (0.037–0.386)	0.000		
III	0.070 (0.015–0.325)	0.001		
IV	0.288 (0.116–0.719)	0.008	1.781 (1.068–2.972)	0.027
<b>CEA</b>				
<5 U/mL	Ref			
>5 U/mL	2.442 (1.095–5.446)	0.029		
<b>CA199</b>				
<35 U/mL	Ref			
>35 U/mL	4.756 (2.075–10.900)	0.000		
<b>ctDNA</b>				
Low	Ref			
high	3.207 (1.461–7.043)	0.004	2.629 (1.017–6.796)	0.046

**Table 4**

Univariate and multivariate Cox regression analyses of variables predicting overall survival time of CRC patients (new assay).

Variables	Univariate analysis		Multivariate analysis	
	Hazard Ratio(95 % CI)	p-Value	Hazard Ratio(95 % CI)	p-Value
<b>Age</b>				
<50	Ref			
50–59	0.000 (0.000–)	0.985		
60–69	0.367 (0.041–3.290)	0.370		
≥70	1.071 (0.313–3.665)	0.913		
<b>Gender</b>				
female	Ref			
male	0.798 (0.253–2.516)	0.700		
<b>Primary lesion site</b>				
distal	Ref			
proximal	0.045 (0.000–2224.018)	0.575		
<b>Tumor size</b>				
<5 cm	Ref			
≥5 cm	0.944 (0.255–3.494)	0.931		
<b>Tumor differentiation</b>				
low	Ref			
Moderate	2.242 (0.316–15.923)	0.420		
high	1.668 (0.354–7.859)	0.518		
<b>TNM classification</b>				
I	Ref			
II	0.053 (0.006–0.451)	0.007		
III	0.000 (0.000–1.459E + 257)	0.963		
IV	0.278 (0.082–0.941)	0.040		
<b>CEA</b>				
<5 U/mL	Ref			
>5 U/mL	3.484 (1.121–10.831)	0.031		
<b>CA199</b>				
<35 U/mL	Ref			
>35 U/mL	11.738 (3.597–38.310)	0.000	12.322 (3.181–47.724)	0.000
<b>ctDNA</b>				
Low	Ref			
high	8.604 (2.322–31.881)	0.001	8.409 (2.084–33.934)	0.003

There were several limitations in this study. Firstly, due to limited number of patients, further studies on larger cohorts are needed to validate our findings. Secondly, longitudinal studies may be performed to further explore the clinical utilities of this mqMSP assay in monitoring and efficacy assessment. It is also interesting to explore whether the blood-based mqMSP assay may be synergistic with other readily available stool-based assays in CRC screening to improve the overall cancer detection rate.

#### CRedit authorship contribution statement

**Dewen Zhu:** Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Jinlei Li:** Writing – original draft, Visualization, Investigation, Data curation. **Wenwen Zhang:** Visualization, Methodology, Data curation. **Yishuai Wang:** Validation, Investigation, Data curation. **Huidong Wang:** Validation, Investigation, Data curation. **Ruoyan Fei:** Validation, Investigation, Data curation. **Qian Ye:** Visualization, Methodology, Data curation. **Danli Peng:** Visualization, Methodology, Data curation. **Ju Luan:** Supervision, Formal analysis, Data curation. **Chang Xu:** Supervision, Formal analysis, Data curation. **Xiaoli Wu:** Supervision, Formal analysis, Data curation. **Dan Huang:** Supervision, Formal analysis, Data curation. **Chunming Ding:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Shengnan Jin:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization.

#### Declaration of competing interest

DZ, JL, CD, and SJ have filed a patent for the relevant markers and assays.

#### Acknowledgements

We would like to thank all the patients who participated in the study and all the support from the doctors and nurses for the collection of samples.

#### Research funding

This work was funded by Wenzhou Key Laboratory of Tumor Molecular Markers (2022HZSY0041), the Leading Innovation and Entrepreneurship Team in Zhejiang Province (Grant 2019R02006), and the National Natural Sciences Foundation of China (Grants 82272404 and 82172358).

#### Informed consent

Informed consent was obtained from all individuals included in this study.

#### Ethical approval

The study was approved by the Clinical Research Ethics of the First Affiliated Hospital of Wenzhou Medical University.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cca.2024.120026>.

#### Data availability

Data will be made available on request.

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