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Title page

Earlier postoperative ctDNA detection predicts recurrence and adjuvant therapy benefit in stage II-III colorectal cancer

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Abstract

Detection of postoperative minimal residual disease (MRD) using circulating tumor DNA (ctDNA) accurately predicts cancer recurrence and may even guide adjuvant chemotherapy. Cost-effective and easily implementable assays capable of detecting MRD early (within days after surgery) will drive wider clinical adaptation. We developed a rapid, single-tube multiplex quantitative methylation-specific PCR (mqMSP) assay targeting 10 rigorously screened methylation markers. In a preoperative cohort, mqMSP achieved 100% specificity in 96 controls, with an overall sensitivity of 73.1% for CRC. In a postoperative cohort of 246 stage II-III CRC patients with a long follow-up time (7-96 months, median: 48 months), we demonstrated that ctDNA detection by mqMSP at an early timepoint (median: 5 days after surgery) predicted worse disease-free survival (HR 7.43, $P < 0.0001$) and overall survival (HR 8.81, $P < 0.0001$). Benefit from completion of recommended adjuvant therapy was only seen for ctDNA-positive patients, but not for ctDNA-negative patients.

Keywords: Colorectal cancer; DNA methylation; circulating tumor DNA; molecular residual disease; recurrence

Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer death in the world [1], with 5-year survival rate at about 65% [2]. The high mortality rate in CRC is predominantly driven by disease recurrence after surgical resection. Therefore, current clinical guidelines recommend adjuvant therapy for stage III and high-risk stage II CRC patients [3-6]. However, the use of adjuvant chemotherapy for stage II patients without high-risk factors remains controversial [7-11]. Better assays for risk stratification and adjuvant therapy guidance are needed.

Circulating tumor DNA (ctDNA)-based detection of postoperative minimal residual disease (MRD) can accurately predict recurrence [12]. Furthermore, for stage II CRC patients, ctDNA-guided treatment reduced adjuvant therapy use without compromising recurrence-free survival and overall survival [13, 14]. Most studies employed a tumor-informed NGS strategy with patient-specific mutations for MRD detection [15-20]. Such approaches are complex (turnaround time 6-8 weeks), difficult to standardize, and in many regions cost prohibitive. Additionally, the first postoperative blood is typically taken 4 weeks after surgery. As a result, the MRD result is only available 10-12 weeks after surgery, which may delay adjuvant therapy decision and compromise treatment outcome [3, 13, 21, 22].

Thus, cost-effective and simple-to-implement assays for MRD detection, preferably capable of analyzing blood samples taken within days after surgery (e.g. prior to discharge after surgery), may greatly benefit a larger proportion of CRC patients. Previous studies from our group and others have demonstrated that PCR-based liquid biopsy targeting DNA methylation markers can predict recurrence in colorectal cancer [23-25]. However, these studies suffered from relatively low specificity in ctDNA detection [24, 25], delayed blood collection (ranging from 3-12 weeks after surgery) [25], potential contamination risk due to DNA pre-amplification [25], and technical complexity in detecting individual markers [25].

We report the development of a new multiplex quantitative methylation-specific PCR (mqMSP) assay targeting 10 rigorously newly selected CRC methylation markers. This mqMSP assay achieved an unparallel specificity for PCR-based liquid biopsy assays, with no false positive detection for blood samples from 96 healthy controls. With a drastically earlier landmark blood sample collection (range: 1-14 days; median: 5 days post-surgery) and a long follow-up time (median, 48 months; range, 7-96 months), we demonstrated that a single timepoint assessment of ctDNA by mqMSP predicts both disease-free survival (DFS) and overall survival (OS). Furthermore, completion of recommended adjuvant therapy showed benefit in ctDNA-positive patients for both DFS (HR = 0.46, 95% CI, 0.22-0.96; $P = 0.02$) and OS (HR = 0.27, 95% CI, 0.10-0.71; $P = 0.004$), but not for ctDNA-negative patients.

Results

DNA Methylation Marker Screening and Analytical Validation of the mqMSP Assay

Detection of MRD may guide the use of adjuvant therapy, or even suggest adjuvant therapy escalation [13, 26, 27]. In such scenarios, it is critical to have high specificity (e.g. over 98%) to avoid unnecessary treatment. At a typical landmark MRD positive rate of 15% for stage II-III CRC patients, an assay with 90% specificity (typically seen for PCR-based assays targeting methylation markers) may generate a false positive rate close to 50%. Due to the low relative abundance (0.01%-0.1%) of ctDNA in the MRD detection context [28], even a very low background methylation from non-malignant cells such as leukocytes may generate false positive signals. Thus, it is critical to screen for DNA methylation markers with extremely high specificity.

Learning from the development and extensive testing of our previously published mqMSP assays [24, 29], we re-screened potential methylation markers for extremely high specificity (Fig. 1A). First, we identified candidate genomic regions by integrating our in-house Reduced Representation Bisulfite Sequencing (RRBS) data with the Cancer Genome Atlas (TCGA) database to select for markers with close-to-zero methylation in buffy coat, very low methylation in normal colorectal tissues, and high methylation in CRC tissues. Subsequently, we tested 67 candidates using Methylation-Sensitive Restriction Enzyme digestion followed by SYBR Green qPCR (MSRE-SYBR-qPCR), followed further by methylation-specific qPCR analysis for 24 prioritized regions. These multi-round screening yielded 10 CRC-specific differentially methylated regions (DMRs), exhibiting hypermethylation in colorectal cancer tissues but drastic hypomethylation in buffy coat and normal colorectal tissues (Fig. 1B). Each of these 10 markers has a prevalence between 70-100% in the tissue samples from 15 advanced adenoma and 40 colorectal cancer patients. When the 10 markers were combined, each sample has at least 3 markers that were methylated (typically 6-8 methylated markers).

Subsequently, a mqMSP assay including 11 assays targeting these 10 DMRs and a control assay targeting the *ACTB* gene were designed. All DMR assays use FAM fluorescence probes while the control assay uses a VIC fluorescence probe. Quantitative PCR was performed in a single-tube format (two duplicate PCR reactions) for all assays using bisulfite-converted DNA. After PCR optimization, we tested the analytical sensitivity of the assay by serially diluted methylated DNA (1%, 0.5%, 0.2%, 0.1%, 0.05%, 0.02%, 0.01% and 0% at a total DNA input of 60 ng). The mqMSP assay can easily distinguish samples with no methylation (negative control) and 0.01% methylation (or about 2 copies for each marker) (Fig. 1C).

Patient Enrollment for Preoperative and Postoperative Cohorts

For the preoperative cohort, 96 healthy volunteers and 122 stage I-IV CRC patients were recruited from two hospitals. All healthy volunteers and 119 patients were included to evaluate the baseline sensitivity and specificity for blood ctDNA detection (Fig. 2A, Supplementary Table S1). For postoperative assessment, 259 patients were enrolled from a single hospital, with 246 patients available for landmark MRD, DFS and OS analysis, of which 214 patients were available for adjuvant chemotherapy benefit analysis (Fig. 2B, Supplementary Table S2). All patients received long-term follow-up (median: 48 months; range: 7-96 months), with non-recurrent patients followed up for a minimum of 24 months (median: 51 months; range: 24-96). During follow-up, 78 patients experienced recurrence and 34 deaths occurred. Patient clinical characteristics are summarized in Table 1.

Performance of the mqMSP Assay in the Preoperative Cohort

Methylation signals from CRC plasma cfDNA were significantly higher than those from healthy controls, with an AUC value of 0.921 (Fig. 1D-1E). The mqMSP assay achieved a specificity of 100% (all 96 healthy controls were negative) and an overall sensitivity of 73.1% (87/119). Sensitivities for stages I to IV were 50% (17/34), 83% (30/36), 74% (26/35) and 100% (9/9) (Supplementary Fig. S1A, Supplementary Table S3). Quantitatively, more advanced stage, T staging, lymph node invasion and metastasis showed significantly higher blood methylation signal (Supplementary Fig. S2A-D). Detection rates also improved with larger tumor size (Supplementary Fig. S1B). Not surprisingly, the performance of CEA (AUC 0.739, sensitivity 36.2%) was drastically lower than mqMSP (Fig. 1E), while the two assays showed some level of synergy in detecting CRC (Supplementary Fig. S1C).

Earlier Landmark Postoperative Blood Test by mqMSP

It is generally believed that surgical trauma may release higher levels of non-malignant cfDNA [30], resulting in even lower proportions of ctDNA. For MRD detection methods targeting somatic mutations,

higher levels of background cfDNA may increase the difficulty in detecting ctDNA. As such, most studies using tumor-informed personalized mutation detection chose one month after surgery as the landmark blood collection timepoint. This late collection of blood, combined with a long (6-8 weeks) turnaround time for the tumor-informed NGS assays, may delay the decision for adjuvant therapy and compromise benefit to patients.

With the rigorously re-screened and validated methylation markers, we hypothesized that mqMSP can be used to detect MRD within days after surgery, as the excessive amounts of non-methylated background DNA is not amplifiable or detectable by the primers and probes highly specific for methylated DNA. We thus collected blood samples from 259 stage II-III CRC patients 1-14 days after surgery (median 5 days). The samples were divided into early (1-4 days) and late group (5-14 days). The early group included 43.2% (51/118) stage II and 56.8% (67/118) stage III patients, whereas the late group included 39.8% (51/128) stage II and 60.2% (77/128) stage III patients. Not surprisingly, total plasma cfDNA concentrations were significantly higher in the early group ($P < 0.05$, Mann-Whitney U test; Supplementary Fig. S3A). However, MRD positive rates were comparable between the groups (22.9% vs. 17.2%; $P = 0.27$, Fisher's exact test; Supplementary Fig. S3C), suggesting that mqMSP may be used for MRD detection using blood samples within days after surgery. To further investigate the effect of sampling time, patients were stratified by the exact postoperative day of blood collection (postoperative days 1-14). The results showed that postoperative sampling time influenced plasma cfDNA levels ($P = 0.02$, Kruskal-Wallis test; Supplementary Fig. S3B), whereas ctDNA methylation signals remained stable ($P = 0.38$, Kruskal-Wallis test; Supplementary Fig. S3D).

Prediction of Disease-free Survival and Overall Survival by ctDNA Using a Single Landmark ctDNA Detection

We further evaluated the prognostic performance of mqMSP. Among 246 patients with evaluable mqMSP results, 49 (19.9%) were ctDNA-positive for the landmark timepoint. These ctDNA-positive patients showed significantly worse disease-free survival (HR = 7.43; 95% CI, 4.76–11.6; $P < 0.0001$, Fig. 3A, Supplementary Table S4 and Supplementary Table S5) and lower overall survival (HR = 8.81; 95% CI, 4.43–17.52; $P < 0.0001$; Fig. 3B). This prognostic association with DFS persisted for both stage II (HR = 15.05; 95% CI, 6.3–36.96; $P < 0.0001$; Fig. 3B) and stage III cohorts (HR = 5.39; 95% CI, 3.17–9.15; $P < 0.0001$; Fig. 3C). Similarly, ctDNA status was significantly associated with OS for both stage II (HR = 23.24; 95% CI, 6.26–86.32; $P < 0.0001$; Fig. 3E) and stage III (HR = 5.24; 95% CI, 2.26–12.16; $P = 0.0001$, Fig. 3F) patients. Among the six dMMR patients in the cohort, two patients with positive MRD experienced recurrence within one year, whereas the other four with negative MRD remained recurrence-free (Supplementary Table S6).

Further multivariate analysis showed that landmark ctDNA status (HR = 5.81; 95% CI, 3.34–10.11; $P < 0.001$), age (HR = 2.40; 95% CI, 1.34–4.28; $P = 0.003$), number of lymph node metastases (HR = 3.91; 95% CI, 1.96–7.79; $P < 0.001$) and elevated postoperative CEA (HR = 3.14; 95% CI, 1.32–7.47; $P = 0.01$) were the significant contributors to DFS (Fig. 4A). Significant difference in DFS comparing stage II and III patients was only observed in ctDNA-negative patients (HR = 2.44; 95% CI,1.23–4.82; $P = 0.01$; Supplementary Fig. S4B), but not in ctDNA-positive patients (HR = 0.96; 95% CI,0.49–1.88; $P = 0.9$; Supplementary Fig. S4A). Multivariate analysis for OS revealed landmark ctDNA was the most significant contributor (HR = 6.27; 95% CI, 2.53–15.53; $P < 0.001$), with lymph node metastases as a minor contributor (HR = 3.63; 95% CI, 1.11–11.81; $P = 0.03$) (Fig. 4B). No significant difference was observed for ctDNA-positive or ctDNA-negative cases when comparing stage II and III patients (Supplementary Fig. S4C-D).

Given the quantitative nature of the mqMSP assay, we subdivided ctDNA-positive cases into ctDNA low and ctDNA high groups using the median methylation signal as the cutoff. Interestingly, ctDNA high group showed highest risk for recurrence, followed by ctDNA low group, and lastly ctDNA-negative group ($P < 0.001$, Supplementary Fig. S5A). Such trend was also seen for OS ($P < 0.001$, Supplementary Fig. S5B), demonstrating both landmark ctDNA positivity and quantitative ctDNA levels were predictive of DFS and OS for stage II and III CRC patients.

We defined clinical sensitivity for recurrence prediction by landmark ctDNA analysis as ctDNA positive rate for patients with recurrence (for a specific time period after surgery such as 12 or 24 months). As such, the recurrence prediction sensitivity for landmark ctDNA analyzed by mqMSP were 73.3% and 64.1% for recurrence within 12 and 24 months, respectively. Consistent with previous results, sensitivity varies among different recurrence sites, with liver metastasis most likely to be detected (Supplementary Fig. S6). Interestingly, the "false negative" (relapsed patients with negative landmark ctDNA) cases showed significantly better DFS (compared to ctDNA-low: HR = 0.58; 95% CI, 0.31–1.07; $P = 0.04$; compared to ctDNA-high: HR = 0.34; 95% CI, 0.16–0.71; $P < 0.001$) and OS (compared to ctDNA-low: HR = 0.41;95% CI, 0.14–1.18; $P = 0.048$; compared to ctDNA-high: HR = 0.29; 95% CI,0.11–0.76; $P = 0.002$) (Supplementary Fig. S7A-B).

Prediction of Adjuvant Therapy Benefit by ctDNA using a single landmark ctDNA detection

To evaluate whether postoperative landmark ctDNA status predicts benefit from adjuvant chemotherapy in stage II-III patients, we stratified patients into completed chemotherapy (≥75% of treatment cycles completed) and incomplete chemotherapy (<75% of treatment cycles or no chemotherapy) subgroups. Chemotherapy completion rates were 58.1% (25/43) in ctDNA-positive and 67.8% (116/171) in ctDNA-negative groups. Significantly improved DFS and OS were only observed in ctDNA-positive patients who

completed therapy versus those who did not (DFS: HR = 0.46; 95% CI, 0.22–0.96; *P* = 0.02; OS: HR = 0.27; 95% CI, 0.10–0.71; *P* = 0.004; Fig. 5A-B). In contrast, no significant survival benefit in DFS or OS was observed in ctDNA-negative patients with chemotherapy completion (DFS: HR = 0.65; 95% CI, 0.33–1.28; *P* = 0.18; OS: HR = 0.35; 95%, CI 0.10–1.12; *P* = 0.06; Fig. 5A-B). These findings suggest that postoperative ctDNA-positive patients are more likely to derive a clinically meaningful benefit from the completion of recommended adjuvant chemotherapy.

Discussion

Recurrence after curative surgery is the leading cause for treatment failure resulting in poor prognosis. Adjuvant therapy based on clinicopathological staging is used to reduce recurrence, while post-surgical monitoring by imaging and protein biomarkers are used for recurrence detection. These approaches have been shown to be useful yet far from sufficient for post-surgical treatment and monitoring [31-33]. Liquid biopsy assays targeting ctDNA markers for MRD detection have been demonstrated as the most accurate approach for predicting recurrence risk, detecting relapse early, and even guiding adjuvant therapy in CRC [15, 34-36]. However, current MRD detection methods targeting somatic DNA mutations suffer from late post-surgical blood collection (one month after surgery) on top of slow turnaround time (6-8 weeks) due to assay complexities, which may delay adjuvant therapy and lead to suboptimal outcome [21, 37]. Additionally, the high cost may prevent such tests to be widely used.

In this study, we tackled a few critical issues in using DNA methylation markers for MRD detection. The first issue is on assay specificity. DNA methylation markers are superior to somatic mutations in terms of being shared by a high proportion of patients of a specific tumor. However, DNA

methylation markers suffer from relatively lower specificity due to residual background methylation from non-malignant cells. To solve this problem, we rigorously re-screened CRC-specific DNA methylation markers to identify 10 markers for the new mqMSP assay. This mqMSP assay achieved 100% specificity in 96 healthy controls. Additionally, the mqMSP assay achieved 97% specificity for patients without adjuvant therapy, and over 98% specificity based on over 700 post-surgical plasma samples from non-recurrent CRC patients (data to be submitted for a separate publication for the FIND trial, NCT05904655).

The second issue is on landmark timepoint for blood collection. While vast majority of published studies used one month after surgery for blood collection to avoid cfDNA spike from surgical trauma, we reasoned that detection of DNA methylation markers is far less susceptible to background cfDNA spike due to the highly selective amplification of methylated DNA by mqMSP. We thus collected blood for our postoperative cohort within days after surgery (conveniently at a time before patient discharge from surgery). Our data demonstrated that mqMSP can detect MRD with high sensitivity and specificity at a much earlier and more convenient landmark timepoint. With a faster turnaround time typically within three days after sample collection, MRD results can be delivered to clinicians within three weeks after surgery for timely therapy and follow-up decisions [3].

The third issue is on assay availability, affordability and robustness. Our mqMSP assay is a single-tube qPCR assay (run in duplicate reactions) using a single blood sample (no tissue sample is needed), with consumable cost below $20. The reaction tube is never opened after PCR, which can greatly reduce contamination and false positives. In our pathology lab, we have analyzed over 2000 postsurgical blood samples with mqMSP over the last two years and we did not observe a single case of suspected contamination.

With these critical issues solved, we enrolled 259 stage II-III CRC patients and collected blood samples at a single timepoint (1-14 days, median 5 days) after surgery. We performed a long follow-up (median 48 months; range: 7-96 months) to critically assess how early landmark timepoint MRD status predicts DFS, OS and adjuvant therapy benefit. All non-recurrent patients were followed up for at least 24 months for a full assessment of clinical sensitivity in predicting recurrence. Positive ctDNA at landmark timepoint predicts significantly worse DFS (HR 7.43) and OS (HR 8.81). Furthermore, quantitative analysis of the ctDNA positive cases revealed that higher levels of ctDNA showed even worse DFS and OS. These results demonstrated both qualitative and quantitative landmark ctDNA levels are strong predictors of DFS and OS for stage II and III CRC. Furthermore, only landmark ctDNA positive patients showed significant benefit in DFS and OS with the completion of recommended adjuvant therapies.

Landmark ctDNA status is the most critical timepoint for patient stratification both in terms of adjuvant therapy decision and follow-up frequencies. While results from this study and others showed improved benefit for ctDNA-positive patients completing recommended adjuvant therapy, these patients still showed drastically worse outcome comparing to ctDNA-negative patients (with or without adjuvant therapy) [26, 38], suggesting treatment escalation, or even new ways of therapy may be needed [27, 39, 40].

Our study has a long follow-up time (median 48 months) and a single time point blood ctDNA analysis. For landmark ctDNA negative patients, the recurrence rate for stage II and III patients were 11% and 28%, respectively. After excluding the stage III ctDNA negative patients without adjuvant therapy or with less than half of the adjuvant therapy regimen completed, the recurrence rate was 24%, which is virtually identical to the Alliance N0147 study (median follow-up time 6.1 years) [41], but substantially higher than two studies with relative short follow-up time (recurrence rates of 12% and

10% for median follow-up of 23 and 21 months, respectively) [25, 26]. Additionally, these false negative ctDNA detection may be caused by less ctDNA release for certain metastatic locations (such as lung) and still-not-sufficient sensitivity of the detection methods, which signifies the importance of longitudinal ctDNA testing and regular imaging follow-ups. Timely and frequent dynamic ctDNA monitoring may also be used to assess treatment efficacy and to detect recurrence earlier leading to better patient outcome (such as increased opportunity for secondary curative surgery as in the FIND Trial [NCT05904665]).

This study demonstrated the clinical performance for risk stratification and adjuvant therapy benefit prediction using a single and early timepoint landmark ctDNA analysis by mqMSP. The main strengths of the study are earlier landmark ctDNA analysis, high specificity, ease of use and low cost for the mqMSP method, and a long follow-up time demonstrating the clinical performance. Despite these strengths, several limitations should be acknowledged. First, this is a single center observational study for the postoperative cohort. Second, ctDNA analysis was performed only for a single timepoint. Longitudinal ctDNA analysis is likely to improve the sensitivity for recurrence prediction and to assess adjuvant therapy efficacy (e.g. whether ctDNA positive patients were converted to ctDNA negative after adjuvant therapy). Third, it is critical to further assess how the mqMSP assay can be used to guide better patient management and adjuvant therapy. We have a few ongoing multi-center prospective studies addressing these issues (e.g. FIND trial [NCT05904665] a prospective multicenter randomized phase III trial evaluating whether ctDNA methylation detection by mqMSP may improve the opportunity of recurrent patients receiving curative-intent treatment).

In summary, this study established the robustness and clinical performance of mqMSP, a single-tube, tumor-agnostic, multiplex methylation assay for early post-surgical (within two weeks after surgery)

ctDNA detection in colorectal cancer. The mqMSP assay robustly detected 0.01% methylation, and with a single landmark analysis of ctDNA identified 73.3% and 64.1% of patients who recurred within 12 and 24 months, respectively, with a specificity estimated over 98% on the per sample basis. Patients with positive ctDNA at the landmark timepoint showed significantly poorer prognosis (DFS and OS) for ctDNA positive patients, and significant benefits (DFS and OS) from the completion of adjuvant therapy. Specifically, the mqMSP offers a cost-effective and easy-to-implement approach for early patient risk assessment and adjuvant therapy decision.

Methods

Study Participants

Two cohorts were recruited for this study. A retrospective case-control preoperative cohort of 96 healthy volunteers and 122 treatment naïve CRC patients (stage I-IV) were enrolled between June 1, 2016 and October 1, 2022. A retrospective postoperative cohort of 259 stage II and III CRC patients who received curative surgery were enrolled between February 1, 2016 and October 1, 2020, with a requirement that all event-free patients were followed up for at least 24 months. CRC patients were confirmed by colonoscopy and pathology diagnosis. Healthy controls were confirmed by no hyperplastic or adenomatous polyps by colonoscopy and absence of any malignancy history. For the postoperative cohort, blood samples were collected within 1-14 days (median, 5 days) after surgery, typically before their discharge. Adjuvant chemotherapy was chosen by physicians and patients who were blinded to ctDNA results. The primary endpoints were disease-free survival (DFS) and overall survival (OS). DFS was defined as the time from surgery to first recurrence (confirmed radiologically or

histologically) or death from any cause. OS was defined as the time from surgery to death from any cause.

Ethics statement

This study was approved by the First Affiliated Hospital Ethics Committee (Approval no. 2016-161) and the Ethics Committee of Wenzhou Central Hospital (Approval no. J2021-02-006). All procedures involving human participants were performed in accordance with the Declaration of Helsinki. All subjects were Asian. All subjects participating in this study signed informed consent.

Sample Processing

Blood samples (10 mL) were collected into EDTA tubes (Apostle; San Jose, CA, USA), followed by prompt separation using a two-step centrifugation as described previously [24]. Plasma and buffy coat were stored at -80 ℃, while tissue samples were stored in liquid nitrogen. Genomic DNA from tissue and buffy coat samples was extracted using QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit (Qiagen; Germantown, MD, USA), respectively, according to manufacturer's protocols. Cell-free DNA (cfDNA) from plasma was isolated with the Apostle MiniMax™ High Efficiency cfDNA Isolation Kit (Apostle; San Jose, CA, USA). All DNA samples were quantified using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA), aliquoted, and stored at −80°C until subsequent analysis [24].

Multiplex quantitative Methylation-specific PCR (mqMSP)

Cell-free DNA (cfDNA) samples were converted by the EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) as described previously [24]. An internal control assay targeting the *ACTB* gene was used to quantify the input DNA amount with a VIC-labelled probe [24]. The mqMSP assay contains 11

assays targeting 10 different DNA methylation markers with FAM-labelled probes. The VIC assay specifically amplifies only bisulfite-converted DNA templates, with minimal amplification efficiency for unconverted or incompletely converted DNA. The mqMSP result was deemed positive if ΔCq (VIC_{mean}-FAM_{mean}) > −3 and negative if ΔCq (VIC_{mean}-FAM_{mean}) ≤ -3. FAM_{mean} and VIC_{mean} were calculated using the two qPCR replicates for each sample.

Statistical Analysis

Categorical variables were compared using χ^2 or Fisher's exact tests. Non-normally distributed continuous variables were analyzed with the Mann-Whitney U test. Diagnostic performance was assessed by receiver operating characteristic (ROC) curve analysis. Survival analyses were performed with the Kaplan Meier method (log rank), with survival rates compared via Fisher's exact test at specified timepoints. All p-values underwent Bonferroni correction for multiple testing. Multivariable Cox regression was used for analyzing DFS/OS prognostic factors (validated by cox.zph), using R v4.1.3 and GraphPad Prism.

Data availability

All data generated or analysed during this study are included in this article and its supplementary information files.

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Contributors

C. D. had full access to all data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. C. D., C. X. and S. J. designed research; W.Z., J.Z., Z.L., L.G., X.G., J.L., F. Z., and J.P. were involved in data acquisition; All authors participated in analysis, and interpretation of data; C. D., W.Z., and J.W. wrote the manuscript; C. D., S. J., C. X., W. Z., J. M., and Z. Y. critically revised the manuscript for important intellectual content; W. Z., J. M., and C.D. performed the statistical analysis; C.D., S. J., and C.X. obtained funding; C.X., X.X, J.Z., J.A., R.M., Z.L., J.Y., C.Z., J.L., F. Z., J.P., and J.Luan provided administrative, technical, or material support; and C. D., C. X. and S. J. supervised the research.

Competing Interests

The Authors declare no Competing Non-Financial Interests but the following Competing Financial Interests: C. D. is a founder for Innovation Biomed., Ltd. J. Luan is an employee of Innovation Biomed., Ltd. C. D., S. J., J. W., Z. Y., and J. Luan have filed patent applications for the mqMSP assay (Application No.: PCT/CN2023/121392, No.: 202380096841.4). The patent applicant is Innovation Biomed., Ltd, and the inventors include C. D., S. J., J. W., Z. Y., and J. Luan. The Chinese application (Application No.: 202380096841.4) has been published and has entered substantive examination. The corresponding applications are currently pending. The patent covers the mqMSP-based ctDNA methylation assay for minimal residual disease detection and recurrence risk assessment. The other authors have no competing financial or non-financial interests to declare.

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Figures legends

Fig. 1 Methylation marker screening and performance of the multiplex quantitative Methylation-Specific PCR (mqMSP). a. Flow chart for DNA methylation screening. Abbreviations: RRBS, reduced-representation bisulfite sequencing; CRC. colorectal cancer; MSRE-SYBR-qPCR, Methylation-Sensitive Restriction Enzyme digestion followed by SYBR Green qPCR. **b.** Methylation levels of 10 candidate markers in paired CRC tissues, adjacent normal tissues, and buffy coat samples measured by MSRE-qPCR. **c.** Assessment of analytical performance for the mqMSP assay. Samples with variable methylation levels (0% to 1%) were generated by spiking methylated HCT15 cell line DNA into unmethylated DNA (a DNA pool from 40 healthy buffy coat samples) to assess the performance of the mqMSP assay. ΔCq represents the quantification cycle difference between the internal control and the methylation targets. **d.** Cell-free DNA methylation levels determined by mqMSP assay in healthy controls (n = 96) and CRC patients (n = 119). Dashed line represents the cut-off value. ****$P < 0.0001$, Mann-Whitney U test. **e.** Receiver operating curve (ROC) analysis of cfDNA methylation and CEA for CRC detection.

Fig. 2 Flowchart of Participant Enrollment. ACT, adjuvant chemotherapy; CEA, carcinoembryonic antigen; CRC, colorectal cancer; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; DFS, disease-free survival; MRD, minimal residual disease; NCCN, National Comprehensive Cancer Network; and OS, overall survival.

Fig. 3 Survival Outcomes stratified by ctDNA Status at a Single Postoperative Landmark Timepoint. Kaplan-Meier curves of DFS and OS stratified by postoperative ctDNA status. For DFS, the ctDNA-positive group exhibited a significantly higher recurrence risk than the ctDNA-negative group in postoperative cohort (**a**), stage II cohort (**b**) and stage III cohort (**c**). B. For OS, the ctDNA-positive group was associated with worse OS than the ctDNA-negative group in postoperative cohort (**d**), stage II cohort (**e**) and stage III cohort (**f**). All *P*-values were determined by log-rank test. The associated tables below show proportions of DFS/OS events at specified follow-up intervals.

Fig. 4 Multivariable Cox Analysis of Disease-Free Survival and Overall Survival by Clinicopathologic Factors and ctDNA Status During Postoperative Days 1-14. Multivariate analysis identified landmark ctDNA status, age, lymph node metastases, and elevated postoperative CEA as significant contributors to DFS (a). For OS, landmark ctDNA was the strongest predictor, with lymph node metastases as a secondary factor (b).

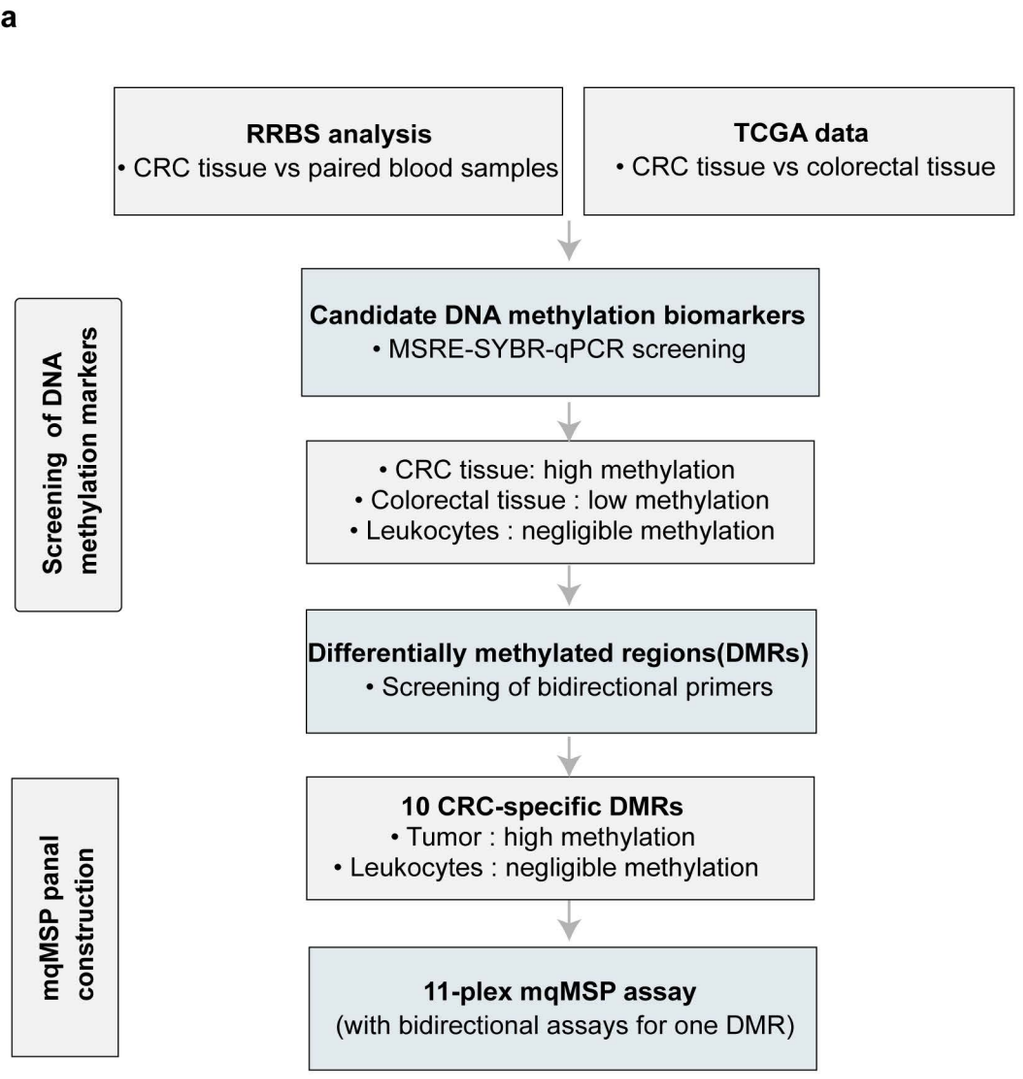
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

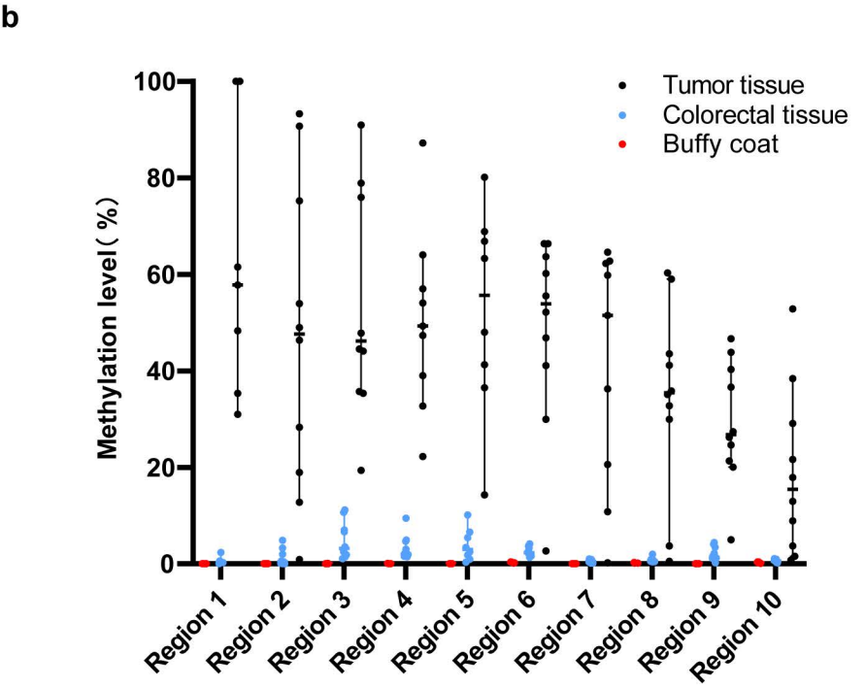
Fig. 5 Patients with Positive Landmark ctDNA Derived Benefit from Completion of Adjuvant Chemotherapy (ACT). Kaplan-Meier curves stratified by ctDNA status and ACT completion. ctDNA (-) and ctDNA (+) indicate negative and positive ctDNA status, respectively. ACT (+) indicates patients who completed adjuvant chemotherapy, while ACT (-) indicates patients with incomplete or no ACT. In the ctDNA-positive cohort, patients with incomplete or no ACT exhibited significantly worse DFS (HR = 0.46; 95% CI, 0.22 – 0.96; *P* = 0.02) and OS (HR = 0.27; 95% CI, 0.10 – 0.71; *P* = 0.004) compared to those with completion of ACT. Conversely, among ctDNA-negative patients, no significant survival differences were observed between ACT (+) and ACT (-) groups for DFS (HR = 0.65; 95% CI, 0.33 – 1.28; *P* = 0.18) or OS (HR = 0.35; 95% CI, 0.10 – 1.12; *P* = 0.06). All *P*-values were determined by log-rank test.

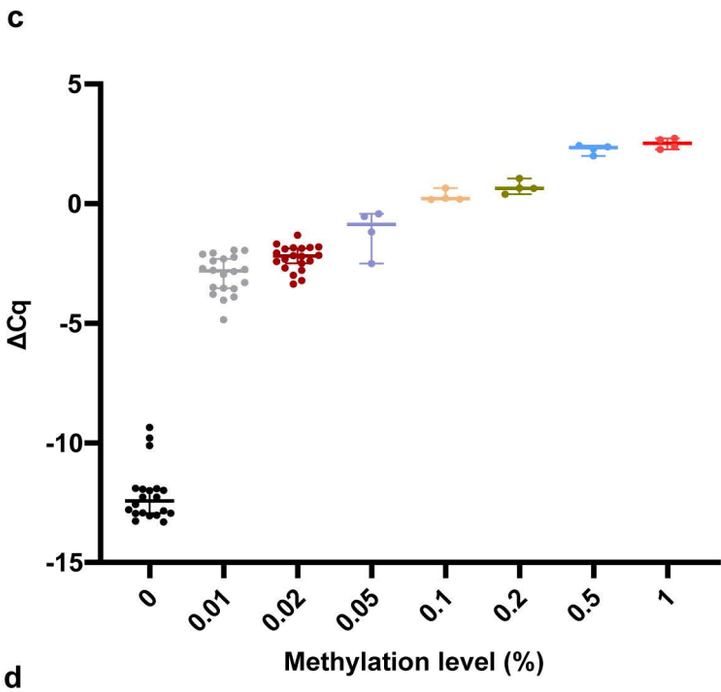
Tables

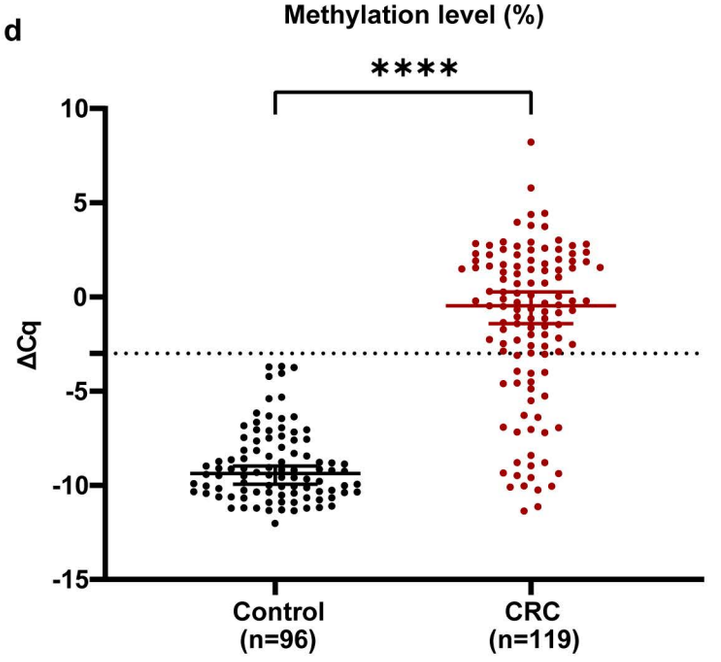
Table 1. Demographic and Clinical Characteristics of Participants.

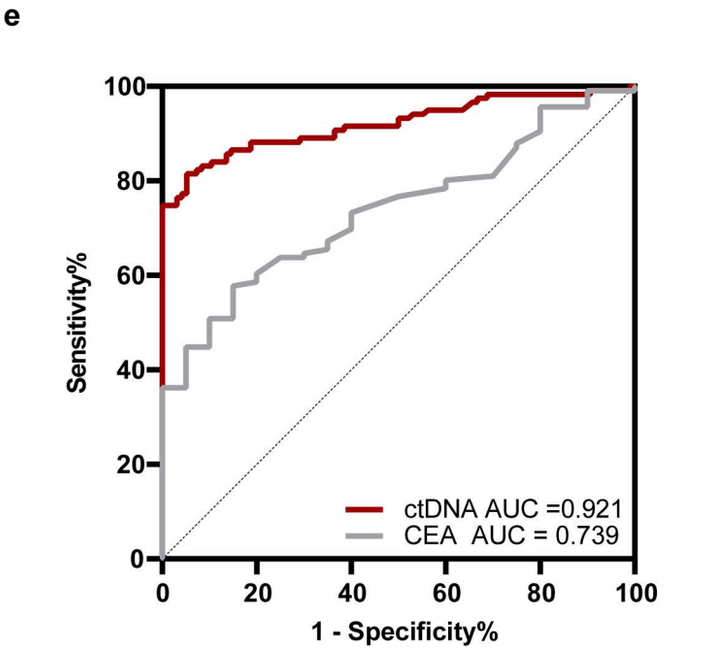
Characteristic	Preoperative cohort		Postoperative cohort
	Control (n=96)	CRC (n=119)	CRC (n=246)
Sex No (%)			
Female	40 (41.7)	60 (50.4)	95 (38.6)
Male	56 (58.3)	59 (49.6)	151 (61.4)
Age No (%)			
<70	83 (86.5)	71 (59.7)	176 (71.5)
≥70	13 (13.5)	48 (40.3)	70 (28.5)
Tumor location No (%)			
Right-side	··	16 (13.4)	38 (15.4)
Left-side	··	98 (82.4)	208 (84.6)
NA	··	5 (4.2)	0 (0)
T stage No (%)			
T1	··	12 (10.1)	2 (0.8)
T2	··	27 (22.7)	22 (8.9)
T3	··	67 (56.3)	152 (61.8)
T4	··	6 (5.0)	70 (28.5)
NA	··	7 (5.9)	0 (0)
N stage No (%)			
N0	··	72 (60.5)	102 (41.5)
N1-N2	··	40 (33.6)	144 (58.5)
NA	··	7 (5.9)	0 (0)
Tumor size No (%)			
0-2 cm	··	16 (13.4)	19 (7.7)
2.1-4 cm	··	50 (42.0)	136 (55.3)
4.1-6 cm	··	29 (24.3)	77 (31.3)
>6 cm	··	17 (14.3)	14 (5.7)
NA	··	7 (5.9)	0 (0)
Stage No (%)			
I	··	34 (28.6)	0 (0)
II	··	36 (30.3)	102 (41.5)
III	··	35 (29.4)	144 (58.5)
IV	··	9 (7.6)	0 (0)
NA	··	5 (4.2)	0 (0)

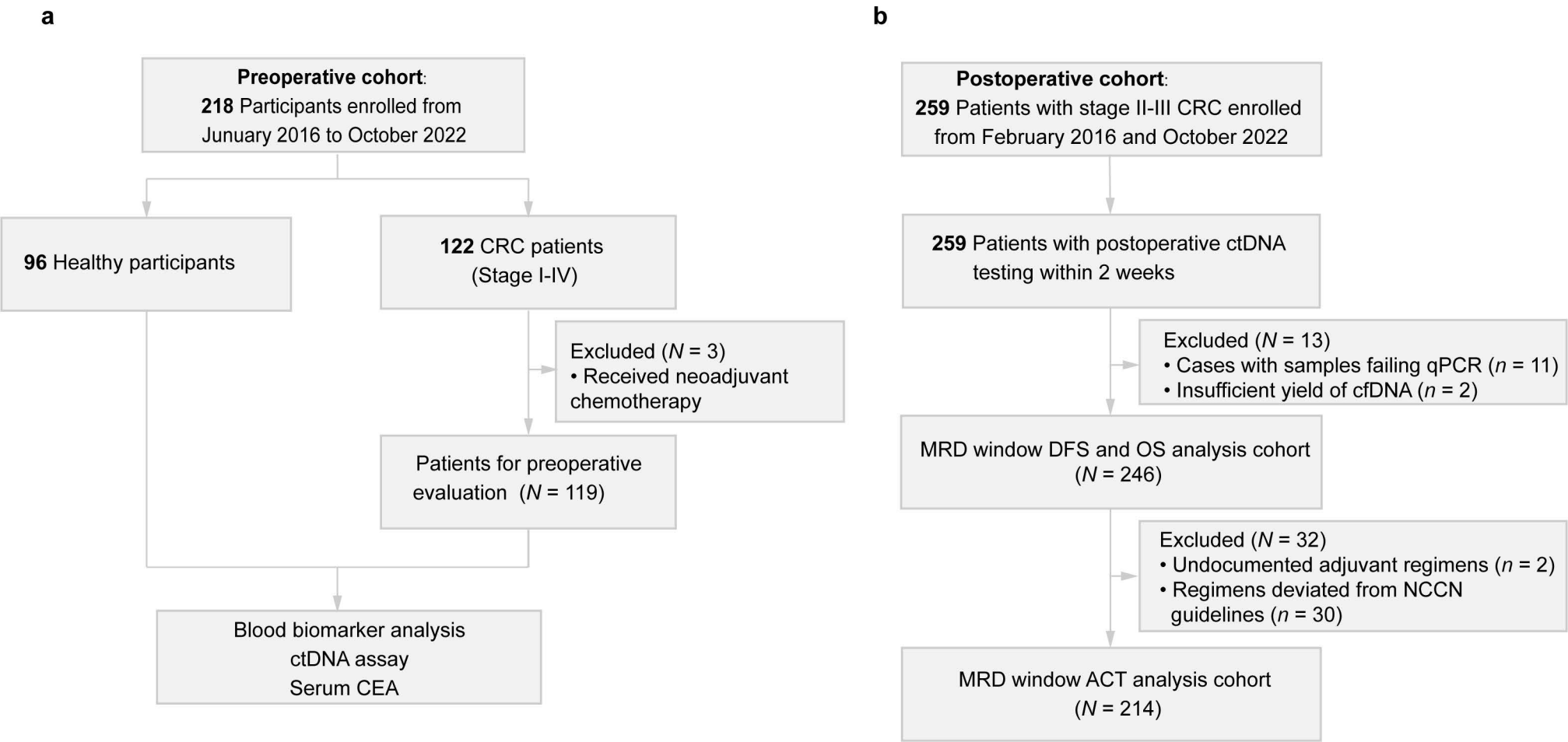

a
RRBS analysis
• CRC tissue vs paired blood samples
TCGA data
• CRC tissue vs colorectal tissue
Screening of DNA methylation markers
Candidate DNA methylation biomarkers
• MSRE-SYBR-qPCR screening
• CRC tissue: high methylation
• Colorectal tissue : low methylation
• Leukocytes : negligible methylation
Differentially methylated regions(DMRs)
• Screening of bidirectional primers
mqMSP panal construction
10 CRC-specific DMRs
• Tumor : high methylation
• Leukocytes : negligible methylation
11-plex mqMSP assay
(with bidirectional assays for one DMR)


b
Tumor tissue
Colorectal tissue
Buffy coat
Methylation level(%)
100
80
60
40
20
0
Region 1
Region 2
Region 3
Region 4
Region 5
Region 6
Region 7
Region 8
Region 9
Region 10

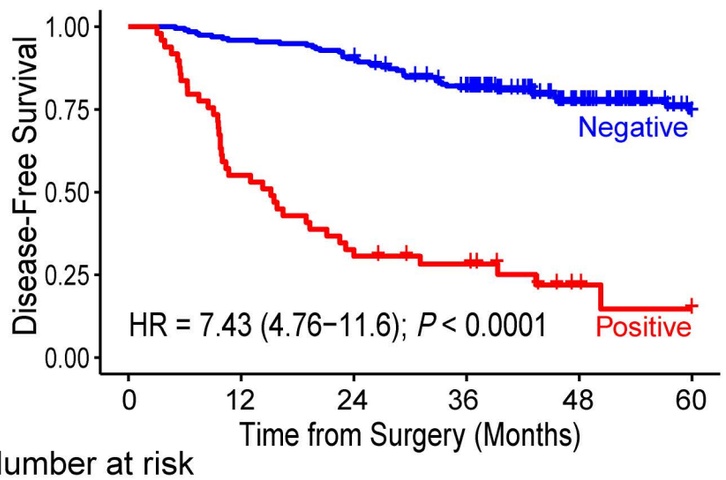

c
ΔCq
5
0
-5
-10
-15
0
0.01
0.02
0.05
0.1
0.2
0.5
1
Methylation level (%)


d
****
ΔCq
10
5
0
-5
-10
-15
Control
(n=96)
CRC
(n=119)


e
Sensitivity%
100
80
60
40
20
0
0
20
40
60
80
100
1 - Specificity%
ctDNA AUC =0.921
CEA AUC = 0.739


a
Preoperative cohort:
218 Participants enrolled from
Junuary 2016 to October 2022
96 Healthy participants
122 CRC patients
(Stage I-IV)
Excluded (N = 3)
• Received neoadjuvant
chemotherapy
Patients for preoperative
evaluation (N = 119)
Blood biomarker analysis
ctDNA assay
Serum CEA
b
Postoperative cohort:
259 Patients with stage II-III CRC enrolled
from February 2016 and October 2022
259 Patients with postoperative ctDNA
testing within 2 weeks
Excluded (N = 13)
• Cases with samples failing qPCR (n = 11)
• Insufficient yield of cfDNA (n = 2)
MRD window DFS and OS analysis cohort
(N = 246)
Excluded (N = 32)
• Undocumented adjuvant regimens (n = 2)
• Regimens deviated from NCCN
guidelines (n = 30)
MRD window ACT analysis cohort
(N = 214)

a

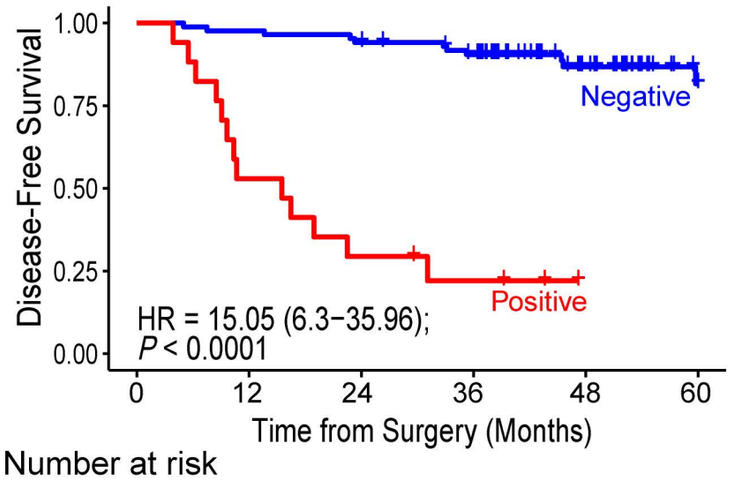


Number at risk

Negative	197	189	178	153	98	43
Positive	49	27	16	12	4	2

ctDNA status	Negative	Positive
Events%	22.3 (44/197)	77.6 (38/49)
12 month DFS% (95% CI)	95.9 (92.0-97.9)	55.1 (40.2-67.7)
24month DFS% (95% CI)	90.4 (85.3-93.7)	30.6 (18.4-43.6)
36month DFS% (95% CI)	81.6 (75.4-86.3)	28.3 (16.5-41.3)

b

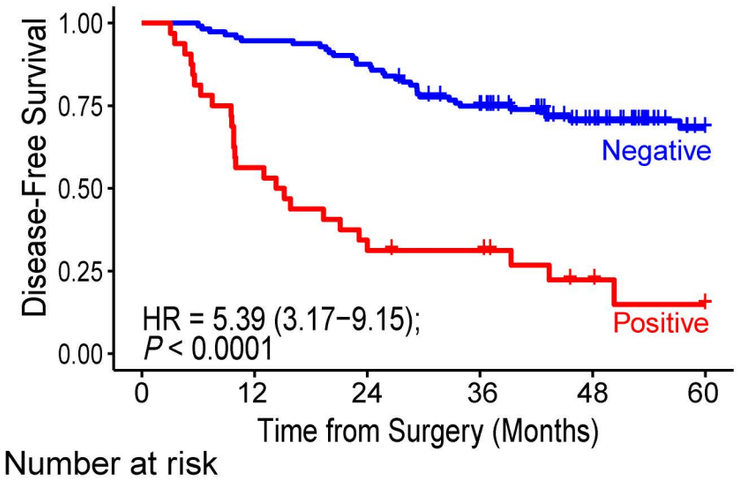


Number at risk

Negative	85	83	80	73	43	16
Positive	17	9	5	3	0	0

ctDNA status	Negative	Positive
Events%	12.9 (11/85)	76.5 (13/17)
12 month DFS% (95% CI)	97.6 (90.9-99.4)	52.9 (27.6-73.0)
24month DFS% (95% CI)	94.1 (86.4-97.5)	29.4 (10.7-51.1)
36month DFS% (95% CI)	90.5 (81.8-95.1)	22.1 (6.1-44.1)

c

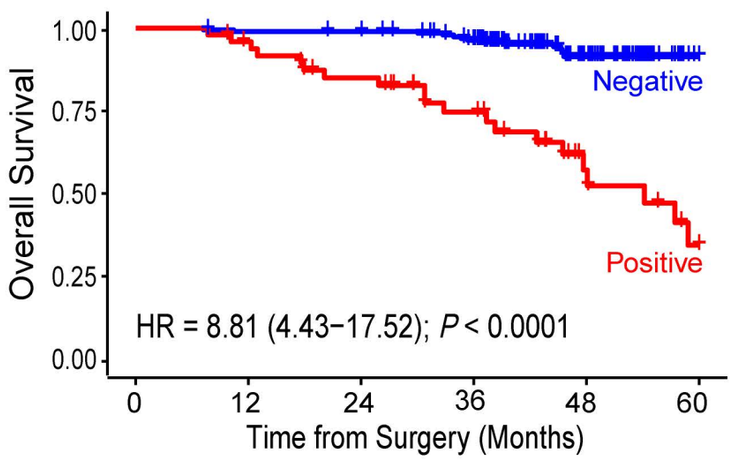


Number at risk

Negative	112	106	98	80	55	27
Positive	32	18	11	9	4	2

ctDNA status	Negative	Positive
Events%	29.5 (33/112)	78.1 (25/32)
12 month DFS% (95% CI)	94.6 (88.5-97.6)	56.2 (37.6-71.3)
24month DFS% (95% CI)	87.5 (79.8-92.4)	31.2 (16.4-47.3)
36month DFS% (95% CI)	74.8 (65.7-81.9)	31.2 (16.4-47.3)

d

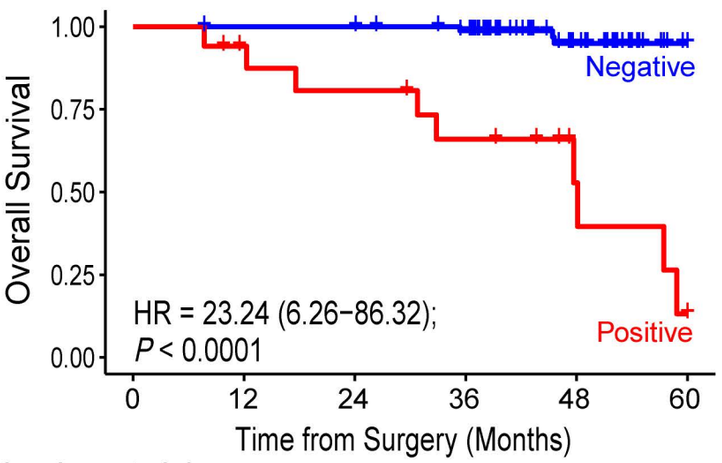


Number at risk

Negative	197	194	193	178	114	51
Positive	49	45	37	27	12	5

ctDNA status	Negative	Positive
Events%	7.11 (14/197)	40.8 (20/49)
12 month OS% (95% CI)	99.0 (96.0-99.7)	95.9 (84.5-99.0)
24 month OS% (95% CI)	99.0 (96.0-99.7)	85.0 (71.1-92.6)
36 month OS% (95% CI)	96.3 (92.5-98.2)	74.6 (58.6-85.2)

e

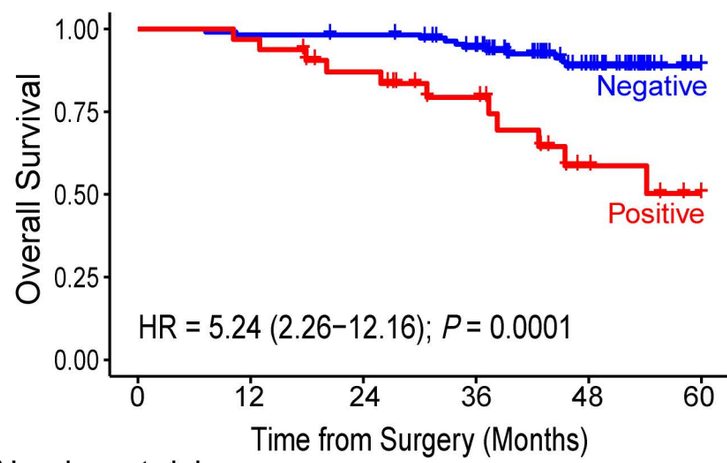


Number at risk

Negative	85	84	84	79	46	18
Positive	17	14	12	9	4	1

ctDNA status	Negative	Positive
Events%	3.53 (3/85)	52.9 (9/17)
12 month OS% (95% CI)	100.0 (NA- NA)	94.1 (65.0-99.1)
24 month OS% (95% CI)	100.0 (NA-NA)	80.7 (51.1-93.4)
36 month OS% (95% CI)	98.8 (91.6-99.8)	66.0 (36.3-84.4)

f

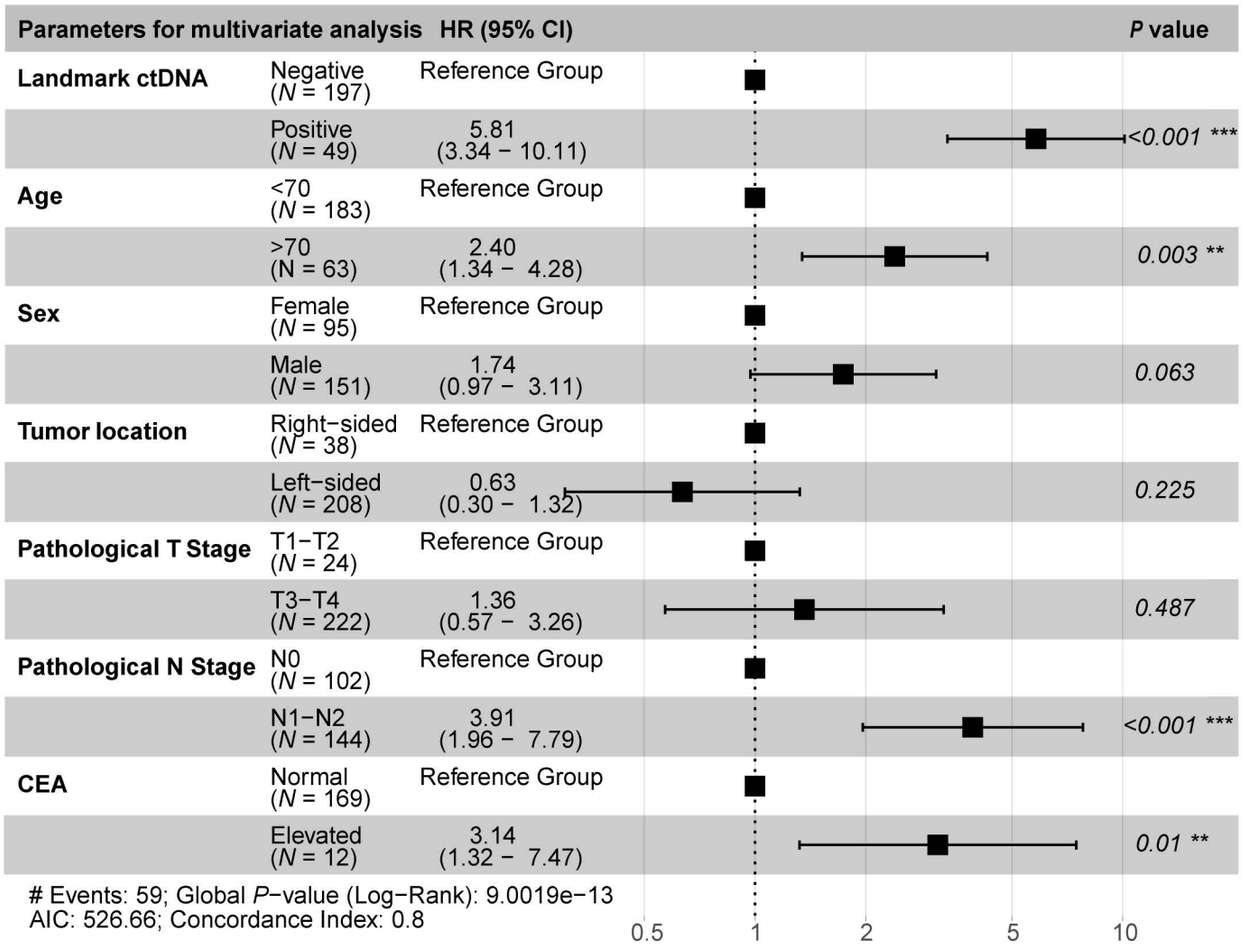


Number at risk

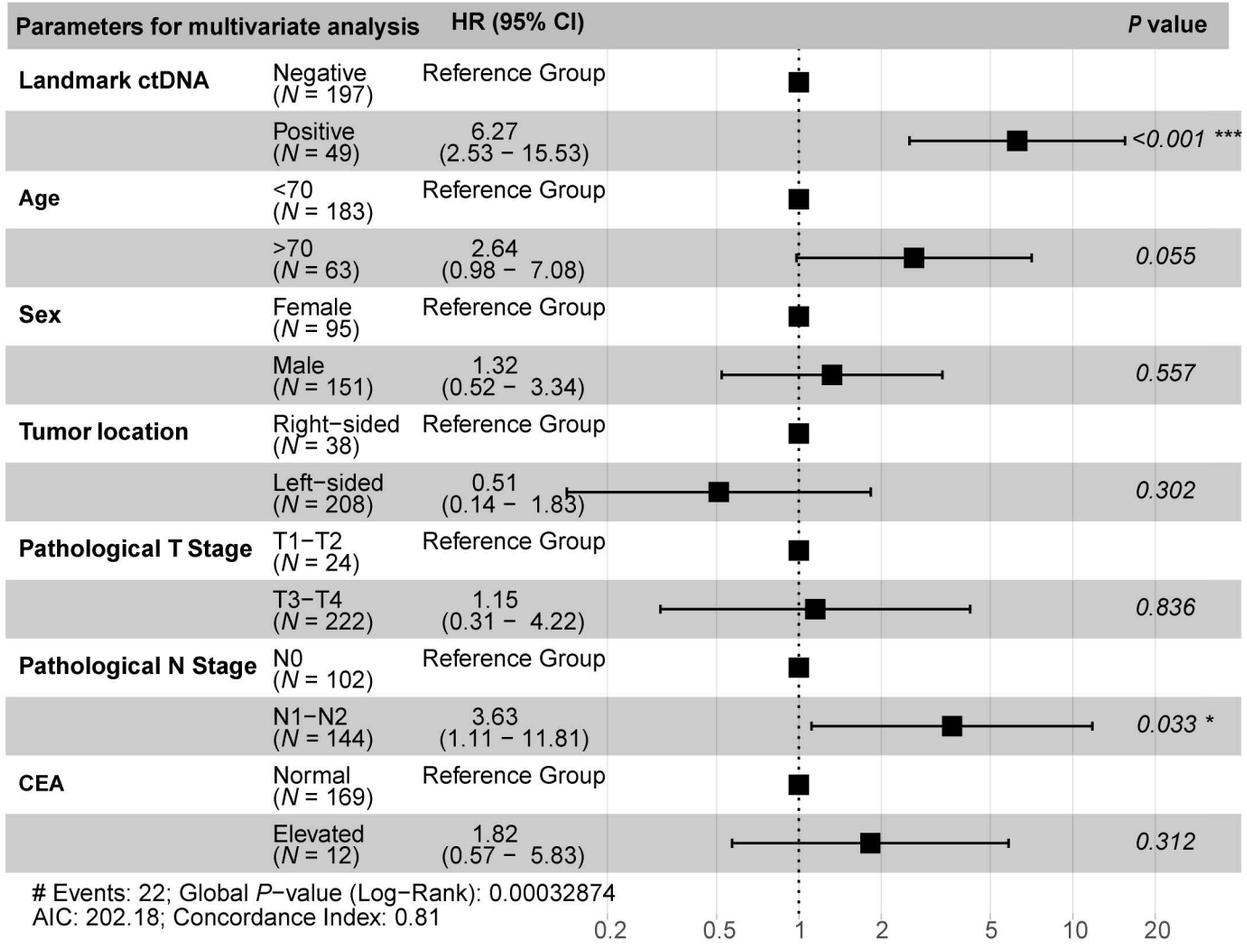
Negative	112	110	109	99	68	33
Positive	32	31	25	18	8	4

ctDNA status	Negative	Positive
Events%	9.82 (11/112)	34.4 (11/32)
12 month OS% (95% CI)	98.2 (93.0-99.6)	96.9 (79.8-99.6)
24 month OS% (95% CI)	98.2 (93.0-99.6)	87.0 (69.0-94.9)
36 month OS% (95% CI)	94.5 (88.2-97.5)	79.4 (59.4-90.3)

a


Multivariate Regression Model for DFS
Parameters for multivariate analysis
HR (95% CI)
P value
Landmark ctDNA
Negative (N = 197)
Reference Group
Positive (N = 49)
5.81 (3.34 − 10.11)
<0.001 ***
Age
<70 (N = 183)
Reference Group
>70 (N = 63)
2.40 (1.34 − 4.28)
0.003 **
Sex
Female (N = 95)
Reference Group
Male (N = 151)
1.74 (0.97 − 3.11)
0.063
Tumor location
Right−sided (N = 38)
Reference Group
Left−sided (N = 208)
0.63 (0.30 − 1.32)
0.225
Pathological T Stage
T1−T2 (N = 24)
Reference Group
T3−T4 (N = 222)
1.36 (0.57 − 3.26)
0.487
Pathological N Stage
N0 (N = 102)
Reference Group
N1−N2 (N = 144)
3.91 (1.96 − 7.79)
<0.001 ***
CEA
Normal (N = 169)
Reference Group
Elevated (N = 12)
3.14 (1.32 − 7.47)
0.01 **
Events: 59; Global P−value (Log−Rank): 9.0019e−13
AIC: 526.66; Concordance Index: 0.8
0.5
1
2
5
10

b


Multivariate Regression Model for OS
Parameters for multivariate analysis
HR (95% CI)
P value
Landmark ctDNA
Negative (N = 197)
Reference Group
Positive (N = 49)
6.27 (2.53 − 15.53)
<0.001 ***
Age
<70 (N = 183)
Reference Group
>70 (N = 63)
2.64 (0.98 − 7.08)
0.055
Sex
Female (N = 95)
Reference Group
Male (N = 151)
1.32 (0.52 − 3.34)
0.557
Tumor location
Right−sided (N = 38)
Reference Group
Left−sided (N = 208)
0.51 (0.14 − 1.83)
0.302
Pathological T Stage
T1−T2 (N = 24)
Reference Group
T3−T4 (N = 222)
1.15 (0.31 − 4.22)
0.836
Pathological N Stage
N0 (N = 102)
Reference Group
N1−N2 (N = 144)
3.63 (1.11 − 11.81)
0.033 *
CEA
Normal (N = 169)
Reference Group
Elevated (N = 12)
1.82 (0.57 − 5.83)
0.312
Events: 22; Global P−value (Log−Rank): 0.00032874
AIC: 202.18; Concordance Index: 0.81
0.2
0.5
1
2
5
10
20