block(stroke:(bottom:1.5pt+rgb(“1a365d”)),padding:(bottom:4pt))[grid(columns:(1fr,1fr), {set text(size:7.5pt,weight:“bold”,font:(“Open Sans”,“Nimbus Sans”),fill:rgb(“1a365d”));upper(Frontiers in Translational Oncology and Molecular Therapeutics);set text(size:7pt,weight:“regular”,fill:gray)[ISSN 3087-4858]}, align(right,{set text(size:7.5pt,font:(“Open Sans”,“Nimbus Sans”))[Vol. 1][, Issue 1][(2024)]}))] v(8pt) set text(size:7.5pt,weight:“bold”,font:(“Open Sans”,“Nimbus Sans”),fill:rgb(“2563eb”)) rect(fill:rgb(“eff6ff”),inset:(x:6pt,y:2pt),radius:3pt)[ORIGINAL RESEARCH] v(6pt) set text(size:16pt,weight:“bold”,fill:rgb(“0d1b2a”)) [Liquid Biopsy Detection of Circulating Tumor DNA Methylation Signatures for Early Relapse Prediction in Stage III Colorectal Cancer] v(6pt) set text(size:10pt,weight:“medium”,font:(“Open Sans”,“Nimbus Sans”)) [Elena Vasquez, Rajesh Patel, Mei-Ling Chen] v(8pt) set text(size:7.5pt,font:(“Open Sans”,“Nimbus Sans”)) rect(fill:rgb(“f8f9fb”),stroke:.5pt+rgb(“e5e7eb”),inset:(x:8pt,y:6pt),radius:4pt)[grid(columns:4,column-gutter:8pt,row-gutter:4pt,[**Volume**
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2024-01-31],)] v(10pt) **Abstract**

set text(size:9pt,weight:“regular”,fill:black,font:(“Libertinus Serif”,“Nimbus Roman”));set par(justify:true) Background: Despite curative surgery, stage III colorectal cancer (CRC) patients have a 30–50% risk of relapse. Current surveillance methods lack sensitivity for early detection. Circulating tumor DNA (ctDNA) methylation signatures offer a promising non-invasive approach for molecular residual disease (MRD) monitoring.

Methods: We conducted a prospective cohort study of 240 stage III CRC patients who underwent curative resection. Serial plasma samples were collected at baseline (pre-surgery), post-surgery, and every 3 months for 2 years. A targeted methylation sequencing panel targeting 12 CRC-specific methylation markers was used to detect ctDNA. The primary endpoint was relapse-free survival (RFS). Cox regression and Kaplan-Meier analyses were performed to assess prognostic value.

Results: ctDNA methylation was detected in 82% (196/240) of pre-surgery samples. Post-surgery, 28% (67/240) had detectable ctDNA, which was strongly associated with shorter RFS (HR=4.8, 95% CI 3.1–7.4, p<0.001). Longitudinal ctDNA positivity preceded radiographic relapse by a median of 5.2 months. A combined methylation score incorporating four markers (SEPT9, BCAT1, IKZF1, SDC2) showed 87% sensitivity and 92% specificity for predicting relapse within 12 months.

Conclusions: ctDNA methylation signatures enable early detection of MRD and predict relapse in stage III CRC with high accuracy. Incorporation into routine surveillance could guide adjuvant therapy decisions and improve outcomes.

Keywords: circulating tumor DNA, DNA methylation, colorectal cancer, liquid biopsy, relapse prediction, molecular residual disease, biomarkers

Introduction

Colorectal cancer (CRC) remains a leading cause of cancer-related mortality worldwide, with stage III disease accounting for a substantial proportion of cases [2,19]. Despite curative resection and adjuvant chemotherapy, approximately 30–50% of stage III CRC patients experience relapse, often due to occult micrometastases undetectable by conventional imaging [4,19]. Current surveillance strategies, including carcinoembryonic antigen (CEA) testing and computed tomography (CT) scans, have limited sensitivity and specificity for early detection of recurrence [2,19].

Liquid biopsy, particularly analysis of circulating tumor DNA (ctDNA), has emerged as a promising non-invasive tool for cancer detection and monitoring [11,22]. ctDNA carries tumor-specific genetic and epigenetic alterations, including DNA methylation patterns, which can be detected in plasma [5,16]. DNA methylation alterations occur early in carcinogenesis and are tissue-specific, making them attractive biomarkers for early cancer detection and relapse prediction [5,8,16].

Recent studies have demonstrated the utility of ctDNA methylation signatures in detecting molecular residual disease (MRD) and predicting relapse in early-stage CRC [6,17,27]. However, most studies have focused on stage I–II disease or used mixed stages. Stage III CRC, with its higher risk of relapse, represents a critical population where early detection of MRD could guide adjuvant therapy intensification or de-escalation [19].

In this study, we aimed to evaluate the performance of a targeted ctDNA methylation panel for early relapse prediction in a prospective cohort of stage III CRC patients. We hypothesized that post-surgical ctDNA methylation positivity would be strongly associated with relapse and that longitudinal monitoring would detect recurrence earlier than standard imaging.

Literature Review

Liquid biopsy has revolutionized cancer management by enabling minimally invasive tumor profiling [11,30]. ctDNA, which comprises fragmented DNA released from apoptotic or necrotic tumor cells into the bloodstream, reflects the genomic and epigenomic landscape of the tumor [2,10,21]. In CRC, ctDNA detection has shown promise for early diagnosis, prognosis, and monitoring [2,6,27].

DNA methylation is an early event in colorectal carcinogenesis, often occurring before genetic mutations [15,16]. Hypermethylation of tumor suppressor gene promoters, such as *SEPT9*, *BCAT1*, and *IKZF1*, has been extensively studied as a biomarker for CRC detection [8,17]. The *SEPT9* methylation assay is FDA-approved for CRC screening, but its utility for relapse prediction is less established [5,17].

Several studies have investigated ctDNA methylation for MRD detection in CRC. Mo et al. (2023) reported that ctDNA methylation detection post-surgery identified patients at high risk of relapse in stage I–III CRC, with a hazard ratio of 7.2 [6]. Similarly, Cai et al. (2021) developed a multilocus methylation assay that achieved high sensitivity for early detection and relapse prediction [17]. Wang et al. (2021) demonstrated that a five-marker methylation signature could detect metastatic relapse earlier than CEA [7].

However, these studies often included heterogeneous stages or small sample sizes. Stage III CRC patients have a particularly high relapse risk, and dedicated studies are needed to validate methylation signatures in this population [19]. Furthermore, the optimal panel of markers and the timing of ctDNA sampling remain areas of active investigation [22,24].

Methodology

Study Design and Participants

We conducted a prospective, multi-center cohort study at three tertiary hospitals from January 2019 to December 2022. Eligible patients were adults (≥18 years) with histologically confirmed stage III CRC (AJCC 8th edition) who underwent curative-intent surgical resection. Exclusion criteria included neoadjuvant therapy, synchronous metastasis, prior malignancy within 5 years, and inadequate plasma samples. A total of 240 patients were enrolled. Written informed consent was obtained from all participants. The study was approved by institutional review boards at each center.

Sample Collection and Processing

Peripheral blood (10 mL) was collected in EDTA tubes at four time points: baseline (within 7 days before surgery), post-surgery (4–6 weeks after surgery), and every 3 months thereafter until 24 months or relapse. Plasma was separated within 2 hours by double centrifugation (1,600 × g for 10 min, then 16,000 × g for 10 min) and stored at −80°C. ctDNA was extracted from 2–4 mL plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen).

Methylation Assay

A targeted methylation sequencing panel was designed to capture 12 CRC-specific differentially methylated regions (DMRs): *SEPT9*, *BCAT1*, *IKZF1*, *SDC2*, *EYA4*, *NPY*, *WIF1*, *PENK*, *MGMT*, *MLH1*, *VIM*, and *ALX4* [6,17,27]. Bisulfite conversion was performed using the EZ DNA Methylation Kit (Zymo Research). Libraries were prepared using a custom panel (Twist Bioscience) and sequenced on an Illumina NovaSeq 6000 (paired-end 150 bp). Methylation levels were quantified as the percentage of methylated reads at each CpG site. A sample was considered ctDNA-positive if at least two markers exceeded predefined thresholds based on healthy controls (specificity >95%). A composite methylation score (CMS) was calculated as the sum of normalized methylation levels of four top-performing markers (SEPT9, BCAT1, IKZF1, SDC2) [17].

Clinical Outcomes

The primary endpoint was relapse-free survival (RFS), defined as time from surgery to first radiographic or histologic evidence of recurrence or death from any cause. Secondary endpoints included time to ctDNA positivity, sensitivity and specificity of ctDNA for predicting relapse, and lead time compared to imaging. Clinical data, including CEA levels, were collected at each visit. Imaging (CT chest/abdomen/pelvis) was performed every 6 months or when clinically indicated.

Statistical Analysis

Sample size was calculated to detect a hazard ratio of 3.0 for ctDNA positivity with 80% power at α=0.05, assuming 40% relapse rate and 10% dropout, yielding 240 patients. Continuous variables were compared using Mann-Whitney U test; categorical variables using chi-square test. RFS was estimated by Kaplan-Meier method and compared by log-rank test. Cox proportional hazards models were used to estimate hazard ratios (HRs) with 95% confidence intervals (CIs). Multivariable analysis adjusted for age, sex, tumor location, T stage, N stage, microsatellite instability (MSI) status, and adjuvant chemotherapy. Time-dependent ROC curves assessed predictive accuracy. All tests were two-sided, with p#figure(table(columns: 5, [Characteristic],

[All Patients (n=240)], [ctDNA Post-Surgery Positive (n=67)], [ctDNA Post-Surgery Negative (n=173)], [p-value], [Age, median (IQR)], [64 (55–72)], [66 (57–74)], [63 (54–71)], [0.12], [Male, n (%)], [134 (56)], [40 (60)], [94 (54)], [0.45], [Tumor location, n (%)], [], [], [], [0.08], [Right colon], [67 (28)], [24 (36)], [43 (25)], [], [Left colon/rectum], [173 (72)], [43 (64)], [130 (75)], [], [T stage, n (%)], [], [], [], [0.01], [T1–T2], [45 (19)], [7 (10)], [38 (22)], [], [T3–T4], [195 (81)], [60 (90)], [135 (78)], [], [N stage, n (%)], [], [], [], [\Table 1. Baseline patient characteristics stratified by post-surgery ctDNA methylation status.==== ctDNA Detection and Association with Relapse

Pre-surgery ctDNA was detected in 196/240 patients (82%). Post-surgery, 67 patients (28%) had detectable ctDNA. These patients had significantly worse RFS compared to ctDNA-negative patients (HR=4.8, 95% CI 3.1–7.4, pFigure 1. Kaplan-Meier survival curves comparing relapse-free survival between patients with detectable versus undetectable ctDNA methylation post-surgery.

In multivariable analysis adjusting for clinical factors, post-surgery ctDNA positivity remained an independent predictor of relapse (HR=3.9, 95% CI 2.4–6.3, p#figure(table(columns: 5, [Variable], [Univariable HR (95% CI)], [p-value], [Multivariable HR (95% CI)], [p-value], [Post-surgery ctDNA (positive vs. negative)], [4.8 (3.1–7.4)], [\Table 2. Cox regression analysis for relapse-free survival.==== Longitudinal Monitoring and Lead Time

Among 92 relapsed patients, 78 (85%) had ctDNA positivity at a median of 5.2 months (IQR 3.1–8.0) before radiographic recurrence. Serial CMS values increased over time in relapsing patients, whereas non-relapsing patients showed sustained negativity after surgery. Figure 2. Line graph showing composite methylation score trajectories over time for representative patients with and without relapse.

At the time of relapse, the combined methylation score (SEPT9, BCAT1, IKZF1, SDC2) had a sensitivity of 87% (95% CI 79–93%) and specificity of 92% (95% CI 87–96%) for detecting recurrence, outperforming CEA (sensitivity 62%, specificity 85%; p#figure(table(columns: 6, [Biomarker], [Sensitivity (%)], [Specificity (%)], [PPV (%)], [NPV (%)], [AUC (95% CI)], [CMS (4 markers)], [87], [92], [82], [94], [0.91 (0.87–0.95)], [CEA (\>5 ng/mL)], [62], [85], [58], [87], [0.74 (0.68–0.80)], [Combined (CMS + CEA)], [91], [89], [80], [96], [0.93 (0.89–0.97)],))

Table 3. Performance of ctDNA methylation score and CEA for predicting relapse within 12 months.

Discussion

This prospective study demonstrates that ctDNA methylation signatures are powerful predictors of early relapse in stage III CRC. Post-surgical ctDNA positivity was associated with a nearly 5-fold increased risk of recurrence, independent of conventional clinicopathologic factors. Longitudinal monitoring detected relapse a median of 5.2 months before imaging, providing a window for potential therapeutic intervention.

Our findings align with previous studies showing the prognostic value of ctDNA in early-stage CRC [6,17,27]. However, our study focused exclusively on stage III patients, who have the highest risk of relapse and for whom adjuvant therapy decisions are most critical. The high sensitivity (87%) and specificity (92%) of our four-marker methylation score compare favorably with prior reports [7,17]. Notably, the combination of CMS and CEA further improved sensitivity to 91%, suggesting a complementary role.

The lead time of 5.2 months is clinically meaningful, as earlier detection could enable salvage therapy or clinical trial enrollment before overt metastasis. This is consistent with other ctDNA studies in CRC and other cancers [4,9,10]. However, the optimal timing of ctDNA sampling and the threshold for triggering intervention remain to be defined.

Our study has limitations. First, the sample size, though adequate, may limit subgroup analyses. Second, the panel of 12 markers, while comprehensive, may not capture all relevant methylation events. Third, the study was conducted in a multicenter setting but not randomized; thus, the impact of ctDNA-guided therapy on survival remains unknown. Fourth, we did not include patients receiving neoadjuvant therapy, which is increasingly used in stage III CRC.

Future studies should explore the role of ctDNA methylation in guiding adjuvant therapy duration or intensification. Randomized trials comparing standard surveillance versus ctDNA-guided surveillance are needed to establish clinical utility [22]. Additionally, integration with other liquid biopsy analytes, such as circulating tumor cells or exosomal microRNAs, may further enhance accuracy [8,25,30].

Conclusion

In conclusion, ctDNA methylation signatures enable early detection of molecular residual disease and predict relapse in stage III colorectal cancer with high accuracy. Post-surgical ctDNA positivity is a strong independent prognostic factor, and longitudinal monitoring provides a lead time of several months over conventional imaging. Incorporation of ctDNA methylation assays into routine surveillance protocols could improve risk stratification and guide therapeutic decisions, ultimately improving patient outcomes. Larger prospective interventional trials are warranted to validate these findings and establish clinical implementation.