Original Article

Evaluation of Molecular Residual Disease by a Fixed Panel in Resectable Colorectal Cancer

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Abstract

Purpose

Molecular residual disease (MRD) is a promising biomarker in colorectal cancer (CRC) for prognosis and guiding treatment, while the whole-exome sequencing (WES) based tumor-informed assay is standard for evaluating MRD based on circulating tumor DNA (ctDNA). In this study, we assessed the feasibility of a fixed-panel for evaluating MRD in CRC.

Materials and Methods

75 patients with resectable stage I-III CRC were enrolled. Tumor tissues obtained by surgery, and pre-operative and post-operative day 7 blood samples were collected. The ctDNA was evaluated using the tumor-agnostic and tumor-informed fixed assays, as well as the WES-based and panel-based personalized assays in randomly selected patients.

Results

The tumor-informed fixed assay had a higher pre-operative positive rate than the tumor-agnostic assay (73.3% vs 57.3%). The pre-op ctDNA status failed to predict disease-free survival (DFS) in either of the fixed assays, while the tumor-informed fixed assay-determined post-op ctDNA positivity was significantly associated with worse DFS (HR, 20.74, 95%CI 7.19–59.83; p<0.001), which was an independent predictor by multivariable analysis (HR, 28.57, 95%CI 7.10–114.9; p<0.001). Sub-cohort analysis indicated the WES-based personalized assay had the highest pre-operative positive rate (95.1%). The two personalized assays and the tumor-informed fixed assay demonstrated same results in post-op landmark (HR, 26.34, 95%CI, 6.01-115.57; p<0.001), outperforming the tumor-agnostic fixed panel (HR, 3.04, 95%CI, 0.94-9.89; p=0.052).

Conclusion

Our study confirmed the prognostic value of the ctDNA positivity at post-op day 7 by the tumor-
informed fixed panel. The tumor-informed fixed panel may be a cost-effective method to evaluate MRD, which warrants further studies in future.

Keywords

ctDNA, MRD, NGS, Tumor-agnostic, Tumor-informed, Fixed panel, Personalized panel
Introduction

Colorectal Cancer (CRC), ranked as the third most prevalent cancer, remains one of the leading causes of cancer-related mortality worldwide [1,2]. Significant advancements in early detection have facilitated the diagnosis of CRC at early tumor stages, enabling more patients to undergo curative resection and experience improved outcomes. In early-stage CRCs, curative resection is often performed, resulting in a remarkable 5-year survival rate surpassing 80% [1,3]. Standard treatment for stage II-III CRC involves postoperative adjuvant chemotherapy (ACT) or follow-up, considering clinical risk factors and mismatch repair (MMR) status after radical surgery. However, over 80% of stage II and around 50% of stage III patients achieve clinical cure through surgery alone, while 15-20% of stage II/III patients experience disease recurrence despite ACT administration [4-6]. These findings highlight the existing dilemma of over- and under-treatment for early-stage CRC. Therefore, there is an imminent necessity for a reliable biomarker to distinguish high-risk patients from those with a favorable prognosis and minimal risk of recurrence. This will enable the implementation of personalized and effective treatment strategies.

Circulating Tumor DNA (ctDNA) detection to assess the presence of molecular residual disease (MRD) in patients after curative-intent surgery has emerged as a promising tool for predicting cancer recurrence [7,8]. Numerous studies have consistently reported the association between ctDNA positivity and poor prognosis, whether after curative surgery or ACT [8]. Moreover, the dynamic changes in ctDNA during ACT have been shown to reflect treatment efficacy, as patients who achieve ctDNA clearance exhibit significantly longer disease-free survival (DFS) compared to those with persistent ctDNA positivity [9]. The groundbreaking DYNAMIC II study revealed the potential of ctDNA-guided approach in clinical practice. Researchers showed that the ctDNA-guided adjuvant therapy was non-inferior
to standard management in terms of 2-year recurrence-free survival (RFS). Additionally, the ctDNA-guided approach led to a reduction in the utilization of ACT compared to the standard-management group (15% vs. 28%). These findings demonstrate the clinical significance of ctDNA-based MRD in predicting cancer recurrence and guiding treatment decisions for patients with CRC after curative treatment [10].

Next-generation sequencing (NGS) has been widely utilized to evaluate ctDNA-MRD in various cancer types. Tumor-agnostic assays targeting specific genes have been used for ctDNA analysis, with a limited detection (LOD) ranging from 0.2% to 0.5% [11]. Meta-analysis has shown that ctDNA fraction is usually lower than 0.01% after curative treatment in different cancers [8]. This suggests that the sensitivity of tumor-agnostic assays may not be accurate enough for MRD detection. To improve sensitivity, genomic mutations from tumor tissues are called prior to detect minute amounts of ctDNA after curative treatment. Zhou et al reported a study in 106 patients with locally advanced rectal cancer who underwent neoadjuvant chemoradiotherapy followed by surgery. Using a 1021-gene liquid biopsy panel with special bioinformatic methods for peak calling and positivity determination, they achieved an improved LOD of 0.03% and found that 40% of stage II and 76.8% of stage III patients were ctDNA positive before the operation. Notably, all patients who achieved a pathologic complete response were tested negative for preoperative ctDNA [12]. Moreover, personalized panels designed based on patients’ specific tumor tissue mutations can further enhance sensitivity by increasing the number of effective sites and sequencing depth. One representative assay utilizing this tumor-informed strategy is the Signatera platform by Natera, which customizes a panel of 16 single nucleotide variants based on whole exome sequencing of tumors. The LOD of Signatera is around 0.01%. Reinert et al. has published a multicenter study involving 130 stage I-III colon cancer patients assessed by the Signatera platform. They reported a
preoperative ctDNA positive rate of 90.6% in stage II-III patients and found that ctDNA-positive patients had a significantly higher risk of recurrence at 30 days post-operation (HR 7.2, 95% CI 2.7–19.0; p <0.001) [13]. Similarly, in the GALAXY study of the CIRCULATE-Japan project that enrolled resectable CRC patients, researchers observed 95% and 18% ctDNA positivity rates before and after the operation, respectively. Additionally, ctDNA status at 4 weeks post-operation was found to be an independent factor to predict recurrence (HR 15.3, p <0.001) [9]. Growing evidences support the important role of MRD in guiding precise and personalized treatment. Although the tumor-informed strategy has been widely accepted and favored for evaluating ctDNA MRD due to its superior sensitivity, several questions remain to be answered. For instance, the appropriate time for post-operative landmark in clinical application is not yet well-established, and the requirements of MRD testing platforms in different clinical scenarios are yet to be distinguished. Furthermore, regarding detection technologies, a unified consensus on assessing different MRD detection strategies and assays (including sensitivity, specificity, positive predictive value, negative predictive value, LOD, etc) has not been reached.

Additionally, for tumor-informed assay, whole-exome sequencing (WES) and whole genome sequencing (WGS) are mostly applied to obtain tumor mutations and are usually followed by personalized panels. However, it remains unclear whether patient-specific mutations from a relatively large CRC fixed panel is sufficient and cost-effective for MRD detection, considering the relatively high release of ctDNA in CRCs. Here we report the results from a prospective study conducted in patients with stage I-III CRC, in which we aimed to evaluate the feasibility of a fixed panel in assessing MRD in CRC by comparing WES and panel-based personalized assays.
Materials and Methods

1. Study Design

76 patients with resectable stage I-III CRC who provided written informed consent between May 2019 and July 2020 were enrolled in this study. To be eligible for enrollment, patients needed to have a pathologically confirmed diagnosis of CRC and were able to provide enough tumor tissues for sequencing. Exclusion criteria included a history of another primary cancer within the previous 3 years or treatment with neoadjuvant chemoradiotherapy.

The histopathological features, clinical characteristics and treatment of the patients were collected. Tumor tissue samples were obtained from each patient during the surgical procedure, and blood samples were collected pre-operation (pre-op) and approximately 7 days post-operation (post-op) for ctDNA analysis. The genomic profiles of both tumor tissues and blood samples were initially assessed using a 41-gene panel through capture-based DNA sequencing. The ctDNA in the blood samples was analyzed using two distinct strategies: a tumor-agnostic and a tumor-informed approach. Additionally, two personalized panels were used in randomly selected 41 patients to detect MRD in the blood samples. Detailed information of the MRD assays are described below, with technical parameters depicted in S1 Table. This study was approved by the ethics committee of the First Affiliated Hospital of Soochow University (No. 2021270), and was performed in accordance with the Declaration of Helsinki.

2. DNA isolation

Genomic DNA of tissue specimens was extracted using QIAamp DNA FFPE tissue kits (Qiagen, Hilden, Germany). The extracted DNA was then quantified using the Qubit 2.0 fluorimeter (Life Technologies, CA). Approximately 10 mL of blood were collected in streck tubes (DNA preserving tubes) and processed within 72 h of collection. Whole blood was
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centrifuged at 2200×g for 10 min at 4°C. After discarding the red blood cells and buffy coat, we centrifuged the plasma at 16,000×g for another 10 min at 4°C. Supernatants were subsequently stored at −80°C until further analysis. cfDNA from plasma was purified using a QIAamp Circulating Nucleic Acid kit according to the manufacturer’s instructions.

3. MRD calling using fixed panel

In our previous report, we introduced the fixed panel ColonCore developed by Burning Rock Biotech in Guangzhou, China [14]. This panel has undergone enhancements, now encompassing the complete exons of 41 genes associated with colon cancer, spanning 213 kb of the human genome (S2 Table). This upgraded version of the ColonCore panel was utilized in the current study. The raw sequencing data analysis and tumor-agnostic somatic mutation calling were conducted using the in-house bioinformatics pipeline, as previously reported. A ctDNA positive status is defined as the detection of any somatic mutation. In the tumor-informed caller, variants were treated as false-positive and were first filtered out if they were not detected in paired tumor tissues. ctDNA analysis was conducted using a previously published algorithm called PROPHET (Burning Rock Biotech, Guangzhou, China) [15]. Briefly, the PROPHET ctDNA libraries were prepared with custom adapters that contain 6 bp unique molecular identifiers (UMIs). Sequencing reads were initially mapped to the human reference genome with consensus reads created and remapped to the hg19 reference. A single-stranded consensus sequence (SCS) approach followed by duplex consensus sequence (DCS) was used to create read families. This algorithm utilizes the maximum likelihood (ML) method by monitoring multiple loci to estimate the ctDNA fraction in plasma samples. In PROPHET, the probability (p) of the read count for each informed mutation, following a Poisson distribution, is calculated. If the p-value is less than 0.05, the mutation is considered significant.
and true-positive. Subsequently, a likelihood ratio test is performed to determine the sample-level p-value. In this context, the tumor-informed fixed panel strategy defines a sample as ctDNA-positive if it possesses at least one significant site and a sample-level p < 0.0005.

4. ctDNA analysis through personalized assays

Tissue samples were subjected to sequencing using a panel that included 520 cancer-related genes (OncoScreen plus, Burning Rock, Guangzhou China). Gene list of the OncoScreen plus panel is reported in S3 Table. Alternatively, whole-exome sequencing (WES) was performed using the Twist Human Core Exome kit (Twist Bioscience, South San Francisco, CA), following the manufacturer’s recommendations. Based on the previously reported screening criteria, two sets of individualized probes were designed according to the mutations detected in the tissue samples, and ctDNA was analyzed using the PROPHET algorithm. Through the WES/panel-based analysis of the tumor and matched white blood cells, patient-specific somatic variants were identified. For the panel-based personalized assay, up to 25 mutation loci were included. Hotspot variants with a variant allele frequency (VAF) ≥0.3% or non-hotspot mutations with a VAF ≥2% were selected. Priority was given to hotspot variants, followed by those with high VAF. ctDNA positivity was defined as having one significant site and a sample-level p <0.0005 or having at least two significant sites and a sample-level p < 0.005. On the other hand, for the WES-based personalized assay, up to 50 highly ranked variants with a VAF above 3.0% were selected. Mutations with high VAF and high-impact were prioritized. Essentially, variants situated in the repetitive regions, regions with a high GC content (> 75%), and homologous regions were filtered out. The biotinylated capture probe pool was produced in-house based on each personalized panel design. ctDNA positivity was defined as having two or more significant sites and a sample-level p <0.005. Detailed information of
the NGS experiments is reported in our previous study [14,15].

5. Statistical analysis

Continuous variables were described with mean (standard deviation, SD) or median (interquartile range, IQR) and categorical variables were described with number (percentage). Statistical analyses were conducted using the R software (version 4.0.3, https://www.r-project.org/). Categorical variables were compared among groups using the Chi-square test or Fisher's exact test, while the Kruskal-Wallis test and Wilcoxon test were employed for comparing differences of continuous variables between multiple or two groups, respectively. To account for confounding factors, multivariable Cox regression analysis was performed. Survival estimates were obtained using Kaplan-Meier analysis, and differences in survival metrics between groups were assessed using the log-rank test. A significance level of p<0.05 was considered statistically significant.

Results

1. Patient Characteristics and genomic profiling of tumor tissues

After excluding one patient with no trackable mutation detected using the target panel in tumor tissues, a total of 75 patients were included in this study for MRD analysis using four different ctDNA-MRD detection assays (Fig. 1). The baseline characteristics of the 75 patients are summarized in Table 1 and the characteristics for each patient are provided in S4 Table. In brief, the median age at diagnosis was 67 years old (IQR, 57–71 years). Of all the patients, 56.0% (42/75) were stage II, and 41.3% (31/75) were stage III. A total of 46 (61.3%) patients received adjuvant therapies after surgery while 29 (38.7%) patients did not. The follow-up information was available for 71 patients (4 patients were missing) with a median follow-up...
time of 1,173 days (range: 1,015–1,239 days). Till their last follow-up, 21 patients (28.0%) experienced disease recurrence, and 12 (16.0%) patients died (Table 1).

Genomic profiling of tumor tissue samples identified a total of 349 somatic mutations in 75 patients, with a median of 4 mutations per patient. Missense mutations were the most common mutation type, followed by stop-gained mutations (Fig. 2A-B). TP53, APC, KRAS, PIK3A, and SMAD4 were the most frequently mutated genes (Fig. 2B), consistent with a previous report in the Chinese CRC population [16] and The Cancer Genome Atlas Program-Colon Adenocarcinoma/Rectum adenocarcinoma (TCGA-COAD/READ) cohort (S5AFig.). KRAS G12D, KRAS G13D, and KRAS G12V were identified as the most frequent variations (S6 Table).

2. Pre-operative ctDNA Positivity Detected by Fixed Assays and Its Prognostic Value

Pre-operative blood samples were collected from 75 patients, and ctDNA status was assessed by a fixed panel targeting 41 CRC-related genes. We evaluated the performance of the tumor-agnostic and tumor-informed fixed assays in detecting pre-operative ctDNA positivity and predicting recurrence. Quality control data of the blood samples including DNA concentrations are reported in S7 Table. The tumor-agnostic and tumor-informed fixed assays identified 43 (57.3%) and 55 (73.3%) preoperative ctDNA positive patients, respectively (S8 Table). Among the 55 baseline ctDNA-positive patients defined by the tumor-informed fixed assay, a total of 178 somatic mutations were detected, with a median of 3 mutations per patient. The most frequently altered genes and sites detected in ctDNA were similar to those observed in tumor tissues (Fig. 2C and 2D). More than half of the mutations (51%) identified from tissue samples were detected in blood samples (S5B Fig.). The tumor-informed fixed assay showed higher ctDNA positive rates than the tumor-agnostic fixed assay stratified by different stages
and tumor sites (Fig. 3A). By contrast, the standard carcinoembryonic antigen (CEA) blood test for CRC screening and recurrence monitoring only yielded positive results in 29.3% (22/75) of the patients (S8 Table). These results strongly suggest that the tumor-informed assay exhibits higher sensitivity in ctDNA detection compared to the tumor-agnostic panel and CEA-based testing in CRC patients.

The prognostic value of ctDNA before curative treatment remains controversial in various cancer types [17-20]. We then examined whether the pre-operative ctDNA status can identify patients at a higher risk of recurrence. We observed no association between disease-free survival (DFS)/overall survival (OS) and preoperative ctDNA status, assessed by either the tumor-agnostic (DFS, HR 1.68, 95% CI 0.68–4.18; p=0.255, S5C Fig.; OS, HR 0.95, 95% CI 0.30–3.01; p=0.934, S5D Fig.) or tumor-informed fixed assays (DFS HR 0.98, 95% CI 0.38–2.53; p=0.967, S5E Fig.; OS HR 0.69, 95% CI 0.21–2.30; p=0.548, S5F Fig.). However, based on the fraction of ctDNA in plasma samples estimated by the maximum likelihood (ML) method, the ctDNA-positive patients were stratified into two subgroups (top 25%: high ctDNA group; bottom 75%: low ctDNA group, cut-off=0.3484%), and the high ctDNA group exhibited poorer DFS (HR 2.73, 95% CI 0.97–7.69; p=0.047, Fig. 3B), indicating that pre-operative ctDNA levels may be a more sensitive prognostic factor than pre-operative ctDNA positivity.

3. Postoperative Landmark ctDNA Predicts Recurrence

To further explore the post-operative ctDNA status and its prognostic value, plasma samples were collected 7 days post-surgery. The landmark MRD status determined by the tumor-informed fixed assay at post-operative day 7 was significantly associated with DFS (HR 20.74, 95% CI 7.19–59.83; p<0.001, Fig. 4A). Among these patients, 7 out of 7 with post-op ctDNA positivity (100%) and 14 out of 64 with post-op ctDNA negativity (21.9%) experienced
relapse during the follow-up (Fig. 4B), yielding a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 78.1%, respectively. Conversely, no significant association was observed for the tumor-agnostic fixed assay with DFS (HR 1.71, 95% CI 0.69−4.20; p=0.239, S9A Fig.).

Multivariable Cox regression analysis showed that tumor-informed ctDNA positivity at post-operative day 7 was an independent risk factor for predicting recurrence after adjusting sex, age, TNM stage and adjuvant therapy (HR, 28.57; 95% CI, 7.10−114.9, Fig. 4C). Furthermore, MRD-positive patients in stage II have a worse prognosis compared to MRD-negative patients in stage III (HR, 6.22, 95% CI 1.46−26.53; p=0.005, S9B Fig.). These results suggest that the post-operative landmark ctDNA status can outperform traditional TNM staging in providing a more effective prognostic stratification.

In the application of ctDNA-MRD tests, interpreting the predictive power for prognosis based on MRD status at the landmark and its time-dependent changes is crucial but often overlooked. To address this gap, we plotted the NPV and PPV of the landmark MRD test over time (Fig. 4D). Our findings demonstrated that the post-op ctDNA negativity determined by the tumor-informed assay initially maintained 100% NPV within 6 months, which gradually decreased thereafter. However, it still remained above 95% within one year. In contrast, the PPV increased to 100% at the 18th month.

Additionally, we examined the characteristics of the ctDNA-negative patients who relapsed during the follow-up, referred to as “Negative-relapsed” (N-RE) patients. Using the tumor-informed fixed assay, we identified 14 out of 71 patients as N-RE. Moreover, we also determined 50 patients who were post-op ctDNA negative and non-recurrent (N-NR), as well as 7 patients who were post-op ctDNA positive and recurrent (P-RE). Further analysis of the clinical features revealed that these N-RE patients had a higher ratio of CEA positivity (S10
Table). Besides, compared to P-RE patients, N-RE patients exhibited a lower pre-operative ctDNA fraction (p=0.0093, Fig. 4E), as well as a lower prevalence of pre-operative ctDNA positivity (57.1% vs 100.0%, S9C Fig.), though no significant difference was observed, suggesting a less amount of ctDNA was released from the tumor cells in N-RE patients. Consistently, the N-RE patients exhibited longer time from surgery to disease recurrence than P-RE patients (p=0.02, Fig. 4F), suggesting the necessity of longitudinal MRD monitoring in these patients.

We further examined whether genomic alterations might contribute to the ability of ctDNA to be released into plasma. Molecular screening of tumor tissues from N-RE patients revealed a trend of fewer genomic alterations with lower maximum allelic fraction (MaxAF, S9D-F Fig.). Previous studies have indicated that tumor cells harboring mutations in cell cycle-related genes were less likely to release DNA [19]. We did observe a higher percentage of patients harboring mutant genes related to cell cycling (including TP53, SMAD4, CHEK2, and ATM) in N-RE than in N-NR and P-RE patients (92.9% vs. 80.0% vs. 71.4%, S9G Fig.). This trend became more pronounced when considering mutations in SMAD4 alone (42.9% vs. 18% vs. 14.3%, S9H Fig.). Collectively, our results indicated that the genomic background of CRC patients may play a role in ctDNA release, potentially leading to false-negative results.

4. Postoperative Landmark ctDNA in Guiding Adjuvant Therapy

In this cohort, 61.3% of patients (46/75) received adjuvant chemotherapy following curative surgery, including 9 patients with FOLFOX, 22 patients with CAPEOX, 3 patients with RALOX, and 8 patients with a single-agent capecitabine. The information regarding the chemotherapy regimen was missing for 4 patients. Consistent with previous studies [21,22], the landmark ctDNA-negative patients did not experience a DFS benefit from adjuvant therapy.
(HR, 0.83; 95% CI, 0.28–2.40; p=0.725, S11A Fig.). In subgroup analysis, stage IIA patients with negative post-op ctDNA did not derive any significant advantage from intensified chemotherapy regimens (p=0.183, S11B Fig.). Similarly, in stage III patients with landmark negative ctDNA, no significant difference in DFS was observed between the patients receiving FOLFOX and CAPEOX regimens (HR, 0.35, 95% CI, 0.04–3.17; p=0.332, S11C Fig.). Due to the limited sample size, no significant difference in DFS was observed between the post-op ctDNA-positive patients with and without adjuvant chemotherapy (HR, 1.41, 95% CI, 0.28–7.12; p=0.673, S11D Fig.).

5. Subgroup ctDNA Analysis Using Two Different Personalized Assays

To facilitate a direct comparison, we assessed the detection sensitivity and prognostic value of two personalized assays using a sub-cohort of 41 randomly selected patients. Tumor tissue samples were analyzed for variants calling using WES or a targeted panel-based approach with 520 genes. We identified a total of 2,022 variants with WES, of which 85.7% were unique to individual patients. The targeted panel assay identified 524 mutations, with 57.3% being individual unique (S12A Fig.). Notably, the WES-based assay covered a higher median number of variants compared to the panel-based assay, regardless of all mutations (51 vs. 11) or individual unique mutations (46 vs. 5).

The WES-based personalized assay exhibited superior sensitivity in the pre-op test across different stages (total, 95.1%; stage I, 100.0%; stage II, 92.0%; stage III, 100.0%), while the tumor agnostic fixed assay identified the fewest cases of pre-op ctDNA positivity (total sensitivity, 70.7%; stage I, 100.0%; stage II, 64.0%; stage III 80.0%, Fig. 5A). The tumor-informed fixed panel and the panel-based personalized assay displayed similar performance with total pre-op ctDNA positivity of 82.9% and 100% for stage I cancers, while the panel-based
personalized assay performed better in stage II (80.0% vs. 76.0%) but less sensitive in stage III diseases (86.7% vs. 93.3%, Fig. 5A). In addition to the 34 patients detected by the tumor-informed fixed assay, the WES-based personalized assay identified additional 5 patients with relatively lower ctDNA fractions due to its lower LOD (Fig. 5B-C). A total of 46 variants were determined in these 5 patients, and only one variant was also covered by tumor-informed fixed assay (Fig. 5D). 32 patients were positive for pre-op ctDNA test according to both the tumor-informed fixed assay and personalized assays, and the WES-based assay detected the most unique variants in these cases (S12B Fig.). These findings suggest that the fixed panel, even with a tumor-informed strategy, does not adequately cover the positive mutant sites, highlighting the advantages of the WES-based personalized strategy for capturing individual mutations.

Consistent with the above results for fixed assays, no association between pre-op ctDNA positivity and DFS was observed by using the personalized assays (panel-based personalized assay, HR, 0.60, 95% CI, 0.16-2.22; p=0.440, S12C Fig.; WES-based personalized assay, HR, 0.81, 95% CI, 0.10-6.34; p=0.837, S12D Fig.). For post-op landmark analysis, the WES-based personalized and panel-based personalized assays had the same results with the tumor-informed fixed panel (HR, 26.34, 95% CI, 6.01-115.57; p<0.001, Fig. 5E-F, S12E Fig.), all better than the tumor-agnostic fixed panel (HR, 3.04, 95% CI, 0.94-9.89; p=0.052, S12F Fig.). Comparing the performance of predicting recurrence, the WES-based and panel-based assays exhibited same NPV and PPV, as did the tumor-informed fixed assay, indicating comparable performances of the ctDNA assays with the tumor-informed strategies. The tumor-agnostic fixed assay exhibited the worst performance (S13 Table). The 7 N-RE patients were tested negative in all four assays, revealing the inherent limitations of these assays in predicting recurrence at the 7-day post-op landmark. Nevertheless, these limitations could potentially be overcome by implementing longitudinal monitoring.
Discussion

cT-DNA-based MRD detection, as an emerging prognostic biomarker, can accurately identify CRC patients at a higher risk of recurrence after curative therapy. While tumor-informed strategies have been recognized for their improved sensitivity in MRD detection, the clinical utility and standardized guidelines for selecting appropriate assays to predict recurrence and guide treatment decisions are still unclear.

In our study, we firstly assessed the prognostic value of the tumor-informed and tumor-agnostic fixed panels. In concordance with previously published studies, we observed that the tumor-informed strategy, even using the same fixed panel with the tumor-agnostic test, yielded improved sensitivity, as indicated by the higher baseline positivity rate (73.3% vs. 57.3%). Although the pre-op cT-DNA status did not show any association with DFS, patients in the pre-op cT-DNA-positive group with elevated cT-DNA levels demonstrated a significantly increased risk of recurrence compared to those with lower cT-DNA levels. This demonstrates that the cT-DNA level, even before the operation, possesses potential predictive value for patient recurrence.

Traditionally, most studies assessing MRD are designed with the postoperative landmark around 1 month after surgery. However, the average hospital stay for CRC patients undergoing resection surgery is typically under 10 days. This extended gap between patient discharge and MRD evaluation necessitates readmission post-discharge, leading to follow-up challenges and potential patient dropout. Furthermore, adhering to NCCN guidelines, clinical practice stresses administering adjuvant chemotherapy (ACT) as soon as medically feasible, while MRD testing entails additional time, potentially delaying the provision of timely cT-DNA results prior to ACT initiation. Given these considerations, early identification and detection of potentially high-risk patients after surgery can greatly assist in decision-making.
and the timely initiation of intensive treatments in postsurgical management. Notably, Chen et al. have highlighted the significance of early ctDNA detection, specifically at 3-7 days after surgery, which might provide novel insights into the post-operative MRD test for colorectal cancer (CRC) management [25]. Taken together, the post-operative day 7 landmark was selected in this study for further exploration. As a result, we have confirmed the significant potential of using tumor-informed fixed assay for identifying patients with poor prognosis. However, a previous study has revealed the association between surgery and elevated cfDNA levels, persisting for up to 4 weeks, which might result a dilution of ctDNA in the blood and introduce false negative results in the early landmark MRD tests [26]. A relatively high N-RE rate was also found in our study. Interestingly, we observed that N-RE patients determined by the tumor-informed fixed assay had a higher ratio of SMAD4 mutation in tumor tissues. A previous research has revealed that there are transcriptional factors take over the regulatory role of SMAD4 when it is mutant, and consequently keep the functional of TGFβ on tumor invasion and metastasis [27]. Additionally, a previous study has discovered that subclones carrying driver mutations related to cell cycling are less likely to release ctDNA [28]. Our results suggest that mutant SMAD4 may impact the ctDNA release or stronger malignancy, which may introduce false negativity. This indicates that the influence of genomic background of cohort should be also considered when evaluating the performance of ctDNA assays.

By using a sub cohort, our study evaluated the ctDNA-MRD using personalized assays with prior WES or fixed panel sequencing on tumor tissues. To our knowledge, this is the first study that performed head-to-head comparisons of these four panels using various strategies and approaches. We observed the highest pre-op ctDNA positive rate with WES-based personalized assays, which indicated its highest sensitivity for ctDNA detection. In the term of post-op, we noticed a similar prognostic capacity with tumor informed personalized assays.
(WES-based or panel-based) and the tumor-informed fixed assay. It could be attributed to the presence of hotspot mutations in CRC that contribute to high ctDNA release. Our results indicate the potential utility of tumor-informed fixed panels in CRC. However, clinical studies with larger sample sizes are required in the future for further validation.

There are also limitations of this study. According to the small cohort, the number of post-op ctDNA-positive patients was not sufficient to observe a significant difference of clinical outcome in patients with or without adjuvant therapy. Additionally, the post-operative day 7 time point chosen may not be optimal, and long-term monitoring was not conducted due to a lack of multiple sampling. Therefore, the median lead-time of MRD detection leading to radioactive relapse can not be measured. Future clinical trials are highly recommended to further assess the clinical utility and benefits for patients using ctDNA-MRD assays employing different strategies and approaches.

Taken together, our study further establishes the prognostic value of ctDNA positivity at post-op day 7 and confirms the prognostic value of the ctDNA positivity at post-op day 7 by the tumor-informed fixed panel. The tumor-informed fixed panel may be a cost-effective method to evaluate MRD in clinic, which warrants further studies in future. These findings may contribute to the advancement of personalized medicine and emphasize the potential of ctDNA-MRD as a reliable biomarker for CRC recurrence prediction.

Our study confirmed the prognostic value of the ctDNA positivity at post-op day 7 by the tumor-informed fixed panel. The tumor-informed fixed panel may be a cost-effective method to evaluate MRD in clinic, which warrants further studies in future.
Ethical Statement

This study was approved by the ethics committee of the First Affiliated Hospital of Soochow University (No. 2021270), and was performed in accordance with the Declaration of Helsinki.

Author contributions

Conceived and designed the analysis: Yang J, Zhou J.

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Conflicts of Interest

The authors declare no potential conflicts of interest, except the employment of Di Peng, Qiaoxia Zhou, Juan Lv, Shuai Fang, Jiaochun Shi, Guoqiang Wang, Shangli Cai and Zhihong Zhang in Burning Rock Biotech.
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References


Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Overall (n=75)</th>
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<tbody>
<tr>
<td><strong>Sex, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>40 (53.3%)</td>
</tr>
<tr>
<td>Male</td>
<td>35 (46.7%)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>65.3 (10.1)</td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>67.0 [57.0, 71.0]</td>
</tr>
<tr>
<td><strong>Tumor site, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Left colon</td>
<td>31 (41.3%)</td>
</tr>
<tr>
<td>Right colon</td>
<td>18 (24.0%)</td>
</tr>
<tr>
<td>Rectum</td>
<td>23 (30.7%)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (4.0%)</td>
</tr>
<tr>
<td><strong>TNM stage, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2 (2.7%)</td>
</tr>
<tr>
<td>II</td>
<td>42 (56.0%)</td>
</tr>
<tr>
<td>III</td>
<td>31 (41.3%)</td>
</tr>
<tr>
<td><strong>dMMR/pMMR, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>dMMR</td>
<td>6 (8.0%)</td>
</tr>
<tr>
<td>pMMR</td>
<td>68 (90.7%)</td>
</tr>
<tr>
<td>Missing</td>
<td>1 (1.3%)</td>
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<tr>
<td><strong>Adjuvant, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>29 (38.7%)</td>
</tr>
<tr>
<td>Yes</td>
<td>46 (61.33%)</td>
</tr>
<tr>
<td><strong>DFS status, n (%)</strong></td>
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<tr>
<td>Disease-free</td>
<td>50 (66.7%)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>21 (28.0%)</td>
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<tr>
<td>Missing</td>
<td>4 (5.3%)</td>
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<tr>
<td><strong>OS status, n (%)</strong></td>
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<tr>
<td>Alive</td>
<td>59 (78.7%)</td>
</tr>
<tr>
<td>Dead</td>
<td>12 (16.0%)</td>
</tr>
<tr>
<td>Missing</td>
<td>4 (5.3%)</td>
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Fig. 1. Study Design.
Fig. 2. Genomic alterations identified from tumor tissues. (A) Heatmap of genetic mutations identified from tumor tissues of 75 patients with detectable mutations. Different types of alterations are presented with colors as illustrated. (B) The most predominate mutation types and the top 10 mutant genes in tissue samples are presented as illustrated. (C) Heatmap of genetic mutations identified from 55 baseline ctDNA-positive patients defined by the tumor-informed fixed assay. (D) The most predominate mutation types and the top 10 mutant genes in blood samples are presented as illustrated.
Fig. 3. Prognostic analysis of pre-operative ctDNA. (A) Boxplots of ctDNA positive rate determined by both tumor-agnostic fixed assay and tumor-informed fixed assay in patients with different stages and different tumor sites. (B) Kaplan-Meier plots of DFS in patients stratified by pre-op ctDNA levels determined by the tumor-informed fixed assay.
Fig. 4. Landmark prognostic analysis of post-operative ctDNA. (A) Kaplan-Meier plot of DFS analysis by post-operative ctDNA status determined by the tumor-informed fixed assay. (B) Boxplot of DFS status in post-operative ctDNA-positive and ctDNA-negative patients determined by the tumor-informed fixed assay. (C) Multivariable analysis of factors associated with recurrence. (D) The dynamic change of PPV and NPV over time by landmark ctDNA status. (E-F) Boxplots of pre-operative ctDNA fraction (E) and time to relapse (F) in post-op positive and relapsed (P-RE) patients and negative but relapsed (N-RE) patients.
Fig. 5. Head-to-head comparison of different fixed and personalized panels in the sub-cohort. (A) Boxplot of pre-op ctDNA positive rate determined by all four assays. (B) Venn diagram of ctDNA positive patients by different assays. (C) Boxplot of ctDNA fractions in positive patients specific determined by WES-based personalized assay and commonly determined by all assays. (D) Positive variants within fixed panel in patients specific determined by WES-based personalized assay. (E-F) Kaplan-Meier plot of DFS analysis by post-op ctDNA status determined by (E) WES-based personalized assay and (F) panel-based personalized assay.