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# Circulating tumor DNA in cancer: A role as a response and monitoring "next-generation" biomarker in cancer therapy

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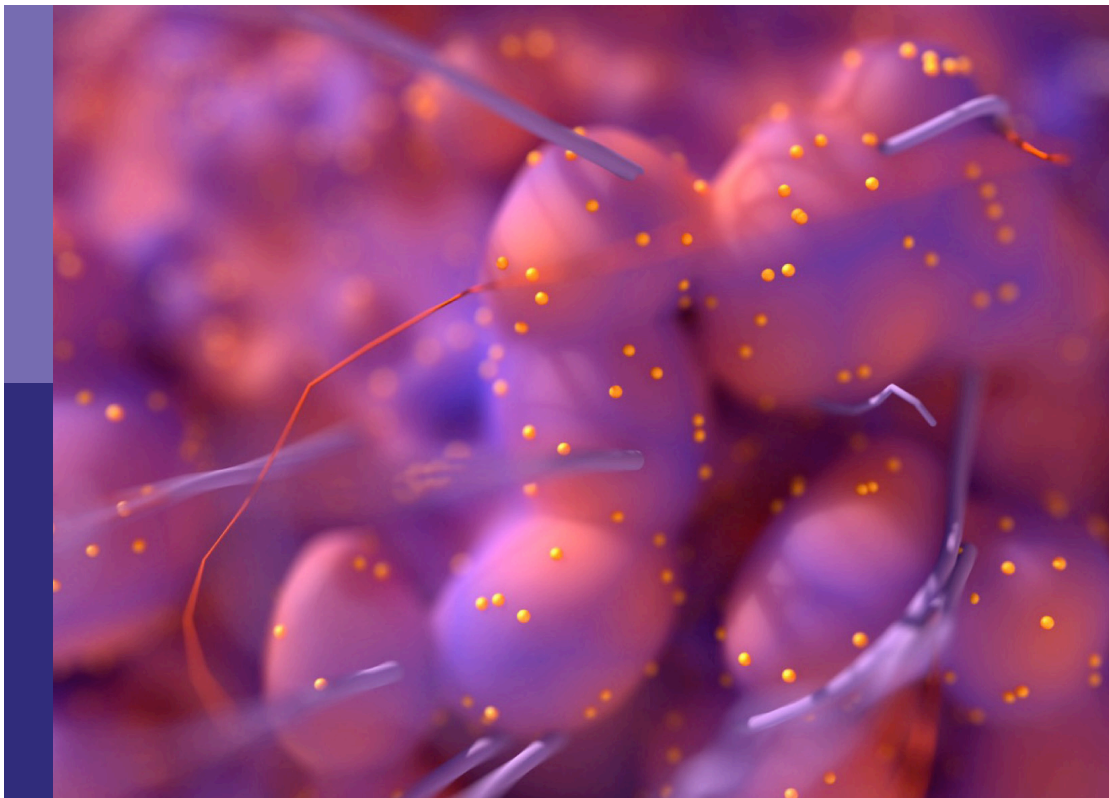
**Published in**
Frontiers in Oncology



ISSN 1664-8714
ISBN 978-2-8325-2727-6
DOI 10.3389/978-2-8325-2727-6

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# Circulating tumor DNA in cancer: A role as a response and monitoring "next-generation" biomarker in cancer therapy

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**Citation**

Latifi-Navid, S., Safaralizadeh, R., Wei, L., eds. (2023). *Circulating tumor DNA in cancer: A role as a response and monitoring "next-generation" biomarker in cancer therapy*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2727-6

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frontiers | Frontiers in Oncology

TYPE Editorial
PUBLISHED 01 June 2023
DOI 10.3389/fonc.2023.1210866



OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 23 April 2023
ACCEPTED 23 May 2023
PUBLISHED 01 June 2023

CITATION
Latifi-Navid S, Safaralizadeh R and Wei L (2023) Editorial: Circulating tumor DNA in cancer: a role as a response and monitoring "next-generation" biomarker in cancer therapy.
*Front. Oncol.* 13:1210866.
doi: 10.3389/fonc.2023.1210866



# Editorial: Circulating tumor DNA in cancer: a role as a response and monitoring "next-generation" biomarker in cancer therapy

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KEYWORDS

**ctDNA, cancer therapy, response, monitoring, biomarker**

Editorial on the Research Topic
Circulating tumor DNA in cancer: a role as a response and monitoring "next-generation" biomarker in cancer therapy

In recent years, circulating tumor DNA (ctDNA) has gained substantial promise as a sensitive biomarker for tumor diagnosis, prognosis and response monitoring of a wide range of treatment modalities. This sensitive biomarker has been shown to be effective in detecting residual disease and diagnosing recurrence, and in tumor-specific adjuvant therapy and targeted therapy (1), Peng et al. A ctDNA biomarker is also innately sensitive and specific for metastatic cancer (2, 3). In this way, ctDNA as a liquid biopsy may represent an exciting era in cancer management, but there remain some challenges. Specifically, we need to 1) learn more about ctDNA's biological characteristics (such as its size, existing form, and mechanism of release), 2) improve the sensitivity of the method for detecting ctDNA, and 3) validate its translation into routine clinical practice through a variety of clinical trials and multi-center cohorts. This Research Topic embodies 10 multidisciplinary manuscripts (original research and critical reviews) focused on multifaceted aspects related to "CtDNA in Cancer".

It is essential to understand ctDNA biology in order to develop techniques that allow its analysis. As a result, Sanchez-Herrero et al. offered an overview of ctDNA biological features, including size and structure, mechanisms of shedding and clearance, and physiopathological factors that influence ctDNA levels. Moreover, Peng et al. discussed the clinical applications and challenges of ctDNA and minimum residual disease (MRD) in solid tumors. An MRD test helps to evaluate the patient's prognosis, treatment response, and recurrence risk. They discussed how ctDNA can be used to monitor MRD in solid tumors, such as breast cancer, lung cancer, and colon cancer. Overall, ctDNA-based MRD detection can improve patient outcomes in cancers and assist in clinical decision-making. In a review article, Lam et al. examined ctDNA as a biomarker for gastro-esophageal, colorectal (CRC), and pancreaticobiliary cancers. They discussed how this biomarker's unique strengths might be used in improving management of gastrointestinal cancers.

During palliative care, ctDNA monitoring can be used to detect and track clonal variants linked to acquired resistance to immune-checkpoint inhibitors and targeted therapies. Moreover, ctDNA may be used to guide therapeutic re-challenge for patients who have taken targeted therapies in the past.

Diefenbach et al. developed an NGS panel to identify melanoma ctDNA that includes 15 top gene mutations including the *TERT* promoter. They analyzed 21 melanoma samples from stage III or IV patients who were either untreated or receiving therapy for their disease. The custom panel detected 14/21 (67%) patients with mutations in *BRAF/NRAS/TERT* promoter, one of whom contained a *TERT* C250T mutation in one negative sample for *BRAF* and *NRAS* mutation. They plan to expand their custom panel to 50 genes in order to improve detection rates of stage IV melanoma to >90%. Liquid biopsy approaches based on ctDNA may be an effective method of interrogating gastrointestinal stromal tumors (GISTs). Ko et al. tested plasma samples from 46 patients with a customized 29-gene Archer® LiquidPlex™ target panel. This is an attractive non-invasive method for obtaining relevant clinical data during disease progression.

Endocrine therapy is a cornerstone of therapy for hormone receptor-positive (HR+), HER2-negative metastatic breast cancer (mBC). Urso et al. evaluated the concordance between ctDNA and *ESR1* status in metastatic tumors. A 91% concordance rate was found between tumor tissue and plasma *ESR1* status. The study showed that liquid biopsy could be an alternative to tissue biopsy for the assessment of *ESR1* mutations in mBC. By sequencing the entire exome of cfDNA, Lee et al. identified novel genetic mutations linked to drug resistance in lung cancer and CRC patients treated with EGFR-targeted therapies and chemotherapy. Sixteen genes in CRC and seven genes in lung cancer were found. Additionally, TTN R7415H and ADAMTS20 S1597P mutations in CRC, as well as the GPR155 I357S mutation in lung cancer, were frequently detected during acquired resistance. This indicates that these mutations play a critical role in acquired resistance to chemotherapy. It is estimated that 3~5% of non-small cell lung cancers (NSCLCs) have leptomeningeal metastases (LM). As indicated by Bai et al., CSF ctDNA from a lung adenocarcinoma patient showed oncogenic mutations before CSF cytology and MRI confirmed LM, indicating CSF ctDNA as a potential early detection tool. A study by Wu et al. examined ctDNA mutated genes, prognosis, and the association between the altered genes in ctDNA and clinical parameters in lymphoma. They proposed that NGS-based analysis of ctDNA mutations can reveal heterogeneities in lymphoma subtypes, which could offer new therapeutic targets, insights into genomic evolution, and new approaches to risk-adaptive therapies.

Interestingly, Chan et al. compared tumor-informed versus tumor-agnostic approaches to ctDNA analyses in CRC patients. The benefits of a single-time point ctDNA analysis were compared with serial monitoring of ctDNA after definitive treatment. They concluded that longitudinal monitoring of tumor-informed ctDNA is highly analytically sensitive, with a low probability of false-positive rate due to clonal hematopoiesis mutations, as well as improved sensitivity to detect recurrence, which may modify CRC clinical management.

Altogether, the original articles and reviews collected in this Research Topic provide original insights and critical perspectives for translating ctDNA into clinical practice and management of patients suffering from malignant tumors.

## Author contributions

The work has been approved for publication by all authors who have made substantial, direct, and intellectual contributions.

## Funding

The work was partially supported by the National Institute for Medical Research Development (NIMAD) Grant No. 958117, Tehran, Iran. The supporter had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

## Acknowledgments

This topic's success would reflect the author's efforts, the reviews and external editors' work, and Frontiers' Editorial Office's invaluable assistance.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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frontiers in Oncology

ORIGINAL RESEARCH
published: 11 March 2021
doi: 10.3389/fonc.2021.625636

# ESR1 Gene Mutation in Hormone Receptor-Positive HER2-Negative Metastatic Breast Cancer Patients: Concordance Between Tumor Tissue and Circulating Tumor DNA Analysis

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**Specialty section:**
This article was submitted to Cancer Molecular Targets and Therapeutics, a section of the journal Frontiers in Oncology

**Received:** 03 November 2020
**Accepted:** 29 January 2021
**Published:** 11 March 2021

**Citation:**
Urso L, Vernaci G, Carlet J, Lo Mele M, Fassan M, Zulato E, Faggioni G, Menichetti A, Di Liso E, Griguolo G, Falci C, Conte P, Indraccolo S, Guarneri V and Dieci MV (2021) ESR1 Gene Mutation in Hormone Receptor-Positive HER2-Negative Metastatic Breast Cancer Patients: Concordance Between Tumor Tissue and Circulating Tumor DNA Analysis. Front. Oncol. 11:625636. doi: 10.3389/fonc.2021.625636

Endocrine therapy represents the cornerstone of treatment in hormone receptor-positive (HR+), HER2-negative metastatic breast cancer (mBC). The natural course of this disease is marked by endocrine resistance, mainly due to Estrogen Receptor 1 (ESR1) acquired mutations. The aim of this study is to evaluate the concordance between ESR1 status in metastatic tumor specimens and matched circulating tumor DNA (ctDNA). Forty-three patients with HR+, HER2-negative mBC underwent both a metastatic tumor biopsy and a liquid biopsy at the time of disease progression. DNA extracted from formalin fixed paraffin embedded (FFPE) tumor specimens and ctDNA from matched plasma were analyzed by droplet digital (dd)PCR for the main ESR1 mutations (Y537S, Y537C, Y537N, D538G, E380Q). We observed a total mutation rate of 21%. We found six mutations on tissue biopsy: Y537S (1), D538G (2), Y537N (1), E380Q (2). Three patients with no mutations in tumor tissue had mutations detected in ctDNA. The total concordance rate between ESR1 status on tumor tissue and plasma was 91%. Our results confirm the potential role of liquid biopsy as a non-invasive alternative to tissue biopsy for ESR1 mutation assessment in mBC patients.

**Keywords: Estrogen Receptor 1 (ESR1), metastatic breast cancer, endocrine therapy, ctDNA, liquid biopsy**

## INTRODUCTION

Hormone receptor-positive (HR+) breast cancer (BC) accounts for about one third of all BC (1). Endocrine manipulation is the mainstay of treatment of HR+/human epidermal growth factor 2- negative (HER2-) BC, and the traditional armamentarium includes aromatase inhibitors (AI), selective estrogen receptor modulators (SERMs, as tamoxifen), selective estrogen receptor degraders (SERDs, as fulvestrant).

However, in the metastatic setting, development of resistance invariably occurs, and about 15–20% of patients show *de novo* resistance (2–4). Several mechanisms have been linked to endocrine resistance, including mutation in Estrogen Receptor 1 (*ESR1*) gene. This gene, located on chromosome 6, encodes for ER$\alpha$, a member of the nuclear hormone receptors superfamily (5). In response to estrogens, ER interacts with specific estrogen response elements (EREs) on DNA and promotes cell proliferation. Moreover, ER harbors numerous bi-directional cross-talks with membrane tyrosine kinase receptors such as epidermal growth factor (EGFR), HER2, insulin-like growth factor (IGFR), that play an important role in breast cancer cells' growth and survival (6–9).

*ESR1* mutations mostly occur in specific hotspots located in the ligand-binding domain of the receptor and result in estrogen-independent function of ER (10). The most common ESR1 mutations are Y537S/N/C and D538G (11).

*ESR1* mutations are rare in primary BC and become more frequent in the metastatic setting, with a total rate of about 30% (12, 13). These mutations are relatively rare in patients treated with tamoxifen only and typically develop after previous exposure to aromatase inhibitors, as a result of the selective pressure of endocrine deprivation therapies (12–18).

Mutant cells are resistant to AI *in vitro*, while high doses of tamoxifen and fulvestrant inhibit signaling of mutant ER (14, 19). In the combined analysis of the SoFEA and EFECT trials, ESR1 mutations have been shown to be associated with worse progression-free survival (PFS) and overall survival (OS) in patients treated with exemestane *versus* fulvestrant, with an objective response rate of 9.5 *versus* 0.0% on respectively fulvestrant and exemestane (17, 20). These findings confirm that ESR1 mutated patients still derive clinical benefit from endocrine therapy with fulvestrant. In this context, preclinical data have shown the effectiveness of new potent oral SERDs (21–24).

Whether detection of ESR1 mutation could impact on treatment decision is still under investigation (NCT03079011).

Circulating tumor DNA (ctDNA) is a cell-free DNA released by tumor cells in the blood (25). ctDNA can be detected in the plasma of patients with cancer, and its analysis may represent a non-invasive tool for detecting and monitoring key gene mutations.

Although different studies showed the potential of Next Generation sequencing (NGS) or Droplet Digital PCR (ddPCR) analyses in identifying ESR1 mutations in ctDNA from HR+ metastatic breast cancer (mBC), few reports compared the sensitivity of the detection in tissue specimens compared to matched plasma samples (26–30).

We conducted a prospective study in a cohort of HR+/HER2- mBC patients to assess the concordance of ESR1 mutation evaluated on matched tumor tissue samples from a metastatic lesion and ctDNA from plasma.

# MATERIALS AND METHODS

## Patients

The study population was represented by a prospective cohort of 43 HR+/HER2- (defined as ER or PgR expression ≥10% and HER2 immunohistochemical 0–1+ or 2+ with no amplification at fluorescence *in situ* hybridization) mBC patients (Age ≥ 18 years) who underwent a biopsy of a metastatic lesion at our Institution, as part of the routine diagnostic-therapeutic management, prior to the start of a new line of systemic treatment. Patients were enrolled from July 2018 to August 2020.

Patients were registered in a prospective database reporting demographics, clinical-pathological features, type of treatment for early (eBC) and advanced BC (aBC), results for ESR1 mutation, and follow-up data.

Treatment for metastatic disease was administered in accordance to national guidelines.

The study (SPIDER) was approved by the Ethic Committee of Istituto Oncologico Veneto (Cod. CESC IOV 2018/26, February 26, 2018). Informed consent was obtained from all subjects.

## Samples Preparation and Analysis

### DNA Extraction From Formalin-Fixed Paraffin-Embedded Tissue Biopsy

We collected 43 FFPE tumor biopsies, reviewed by a pathologist (MF) for tumor tissue quality and quantity. Genomic DNA (gDNA) was extracted from five FFPE sections containing at least 30% of tumor cells using QIAmp® DNA Micro Kit (Qiagen) following the manufacturer's instructions. DNA was quantified by Nanodrop One (Thermo Scientific®). Twenty ng of total gDNA was used for the detection of ESR1 mutations.

### Plasma Sample Collection and DNA Extraction

Liquid biopsy was performed at the same time point of tissue collection, simultaneously with the routine blood exams, with no additional venipuncture. Twenty ml of blood samples was collected in two Helix ctDNA Stabilization tubes (Diatech Pharmacogenetics SRL) and processed within 24 h. Plasma was separated by centrifugation at 2,000 × g for 10 min at 4°C. Next, to further purify plasma from corpuscular cells, the supernatant was centrifuged at 20,000 × g for 10 min at 4°C. Plasma was stored at −80°C until analysis. ctDNA was extracted from 2 ml of plasma using the Maxwell® RSC ccfDNA Plasma Kit (Promega, Madison, Wisconsin, USA), and quantified using Qubit dsDNA HS Assay Kit (Life Technologies, USA). 7.5 µl of ctDNA was used for the detection of ESR1 mutations.

## Detection of ESR1 Mutations by ddPCR

ESR1 mutations were analyzed by ddPCR on the QX200 ddPCR system (Bio-Rad Laboratories) following the manufacturer's instructions. We assessed the following hotspots in 43 tissue specimens: Y537S, Y537C, Y537N, D538G, E380Q. These hotspots were selected based on their frequency among ESR1 mutations in published studies (11) and in the COSMIC dataset (v92). ddPCR probes were purchased from Bio-Rad. To set-up the method, each assay was tested using pSEPT plasmid bearing the indicated mutations. All samples had adequate proportion of tumor cells. All the mutations detected in tumor tissue biopsy were checked in matched ctDNA. As Y537C/N was barely detected in our tissue sample cohort (0.0 and 2.3%, respectively), all plasma samples were analyzed for the three main hotspots: Y537S, D538G, E380Q. Each sample of tumor

tissue DNA was run in duplicate; each sample of cfDNA was run in triplicate.

We defined a positive mutation in tissue DNA with a threshold of 1% allele frequency to avoid technical biases from fixation process (31); for ctDNA we used a cutoff of three mutant-positive droplets per well, following the manufacturer guidelines. Allele frequency for each mutation was determined considering fractional abundance of mutated droplets above the total.

## Sample Size and Statistical Analysis

Statistical analyses were conducted using IBM SPSS (Version 20) Software. The association between categorical variables was evaluated using the $\chi 2$ test.

We evaluated the concordance between ESR1 mutation analysis on matched tissue DNA and ctDNA samples. We considered three concordance measures: i) the rate of ctDNA mutated samples over the total of tumor tissue mutated samples (ctDNA confirmation rate), ii) the rate of concordant mutated matched pairs over the total of pairs showing at least one mutated sample (ctDNA, tumor tissue, or both; concordance mutation rate), and iii) the rate of concordant mutated or concordant wild-type matched pairs over the total of 43 analyzed pairs (total concordance rate).

PFS was calculated as the time interval from the date of liquid biopsy to disease progression or death, whichever was first. OS was calculated from the date of liquid biopsy to death. Patients without an event were censored at the date of last follow-up.

Survival curves were estimated using the Kaplan–Meier model and we used the log-rank test to study differences between groups. For all the performed tests, significance was inferred for a value $p < 0.05$.

# RESULTS

## Patients' Characteristics

From July 2018 to August 2020 we enrolled 43 patients.

Clinicopathologic characteristics at the time of first breast cancer diagnosis are reported in **Table 1**. Seventeen patients had a stage IV *de novo* disease at the time of first diagnosis. All patients had HR+/HER2- tumor phenotype as defined by the protocol on at least one tumor biopsy (either primary tumor or relapse). Among those patients who experienced a disease relapse after a prior diagnosis of primary breast cancer, all but three had a concordant HR-positive and HER2-negative tumor phenotype on both primary tumor and relapse biopsy. Three patients with HER2-positive (n = 2) and triple negative (n = 1) primary breast cancer had a subsequent relapse biopsy showing HR-positive and HER2-negative tumor phenotype. For one patient with HR+/HER2− phenotype on relapse biopsy, receptor status of the primary tumor was not available (**Supplementary Tables 2 if 3**). Median age at first breast cancer diagnosis was 50 years (range 42–62). Most patients had a tumor of ductal histology (n = 37, 86%) and histologic grade 3 (n = 24, 56%). Treatments for early breast cancer are listed in **Table 1**.

**TABLE 1** | Clinical-pathological characteristics at diagnosis and treatment for eBC.

| | | | N (%) |
|---|---|---|---|
| Age (median) | | | 50 (42-62) |
| Menopause | Yes | | 25 (58) |
| | No | | 18 (42) |
| Tumor histotype | Ductal | | 37 (86) |
| | Lobular | | 4 (9) |
| | Other | | 2 (5) |
| Estrogen receptor | Positive | | 39 (91) |
| | Negative | | 3 (7) |
| | NA | | 1 (2) |
| Progesteron receptor | Positive | | 34 (79) |
| | Negative | | 8 (19) |
| | NA | | 1 (2) |
| Histologic Grade | 1 | | 1 (2) |
| | 2 | | 15 (35) |
| | 3 | | 24 (56) |
| | NA | | 3 (7) |
| HER2 | Positive | | 2 (5) |
| | Negative | | 39 (90) |
| | NA | | 2 (5) |
| Stage (AJCC) | I | | 7 (16) |
| | II | | 9 (21) |
| | III | | 9 (21) |
| | IV | | 17 (40) |
| | NA | | 1 (2) |
| CT for eBC | Yes | | 23 (53) |
| | No | | 20 (47) |
| HT for eBC | Yes | | 22 (51) |
| | | Tam | 5 (23) |
| | | AI | 9 (41) |
| | | Tam + AI | 7 (32) |
| | | NA | 1 (4) |
| | No | | 21 (49) |

*N, number of patients; HER2, human epidermal growth factor receptor 2; CT, chemotherapy; HT, hormone therapy; eBC, early breast cancer.*

Patients' characteristics at the time of enrollment in this study are reported in **Table 2**. The majority of patients presented with visceral metastases (n = 29, 67%), and half of the patients had more than three metastatic sites involved (n = 22, 51%) (**Supplementary Table 4**). Twenty-eight patients (65%) had not received any prior systemic therapy for advanced disease at the time of enrolment; 26 patients had been previously exposed to AIs for the treatment of early and/or advanced disease (60%).

## Concordance of ESR1 Mutation on Tumor Tissue Biopsies and ctDNA

We identified the following ESR1 mutations in six of 43 patients (14%) on DNA extracted from tumor biopsies: Y537S (one subject), Y537N (one subject) E380Q (two subjects), D538G (two subjects). Four of the six mutations were confirmed on ctDNA (ctDNA confirmation rate: 67%) (**Supplementary Figure 1**). The low concentration of cfDNA in the two discordant cases, with less than 700 total droplets detected in the plasma, probably reduced the sensitivity of the test.

In order to verify that identified mutations were acquired *de novo*, we tested four out of six matched primary tumors, and no mutations were found (data not shown).

The most frequent ESR1 mutations (Y537S, D538G, and E380Q) were assessed on all ctDNA samples. For three WT

**TABLE 2** | Patients' characteristics according to ESR1 status.

| | | ESR1 (tissue and/or ctDNA) | | | |
|---|---|---|---|---|---|
| | | Total N (%) | WT N (%) | Mut N (%) | P value |
| Visceral metastasis | Yes | 29 (67) | 21 (62) | 8 (89) | 0.123 |
| | No | 14 (33) | 13 (38) | 1 (11) | |
| N° metastatic sites | <3 | 21 (49) | 16 (47) | 5 (56) | 0.650 |
| | ≥3 | 22 (51) | 18 (53) | 4 (44) | |
| LDH | Low | 1 (4) | 1 (5) | 0 | 0.091 |
| | Normal | 13 (54) | 13 (62) | 0 | |
| | High | 10 (42) | 7 (33) | 3 (100) | |
| Previous systemic therapies for advanced disease | 0 | 28 (65) | 25 (73) | 3 (33) | 0.77 |
| | 1–2 | 7 (16) | 4 (12) | 3 (33) | |
| | ≥3 | 8 (19) | 5 (15) | 3 (34) | |
| Prior exposure to AI (eBC and/or aBC) | Yes | 26 (62) | 17 (52) | 9 (100) | **0.010** |
| | No | 16 (38) | 16 (48) | 0 | |
| Prior exposure to CT (eBC and/or aBC) | Yes | 11 (26) | 7 (21) | 4 (44) | 0.160 |
| | No | 32 (74) | 27 (79) | 5 (56) | |
| Prior exposure to Tam only (eBC and/or aBC) | Yes | 5 (24) | 3 (21) | 2 (29) | 0.717 |
| | No | 16 (76) | 11 (79) | 5 (71) | |
| Prior exposure to AI for aBC | Yes | 13 (30) | 7 (21) | 6 (67) | **0.008** |
| | No | 30 (70) | 27 (79) | 3 (33) | |
| Prior exposure to Fulvestrant for aBC | Yes | 9 (21) | 4 (12) | 5 (56) | **0.004** |
| | No | 34 (79) | 30 (88) | 4 (44) | |
| Prior exposure to CDK 4/6 inh for aBC | Yes | 9 (21) | 5 (15) | 4 (44) | 0.051 |
| | No | 34 (79) | 29 (85) | 5 (56) | |
| Prior exposure to everolimus for aBC | Yes | 5 (12) | 2 (6) | 3 (33) | **0.022** |
| | No | 38 (88) | 32 (94) | 6 (67) | |

*Bold values are statistically significant.*

*N, number of patients; WT, wild-type; Mut, mutant; LDH, lactate dehydrogenase; AI, aromatase inhibitor; eBC, early Breast Cancer; aBC, advanced Breast Cancer; CT, chemotherapy; Tam, tamoxifen; CDK, cyclin-dependent kinase.*

*Bold values are statistically significant.*

tissue samples, the analysis of matched plasma revealed the D538G mutation in ctDNA, one of these ctDNA samples showed two concomitant hotspot mutations (D538G and Y537S). Two of these three patients with ESR1 mutation detected on ctDNA and not on tumor tissue had a high disease burden, with more than three metastatic sites and visceral involvement.

**Figure 1** shows the results of ddPCR for the discordant cases between tissue and plasma samples.

Our total ESR1 mutation rate, considering cases showing a mutation on tumor tissue and/or ctDNA over the total, was 21% (9/43).

The concordance rate for mutation was 44% (four cases with ESR1 mutation on matched tissue and ctDNA over nine cases with a mutation detected on tissue and/or ctDNA samples). The total concordance rate (considering the Y537S, D538G and E380Q) between tumor tissue and plasma was 91% (39 concordant mutated or concordant wild-type matched pairs over 43 total pairs analyzed, **Figure 2** and **Supplementary Table 1**).

## Association of ESR1 Mutations With Patients' Characteristics and Previous Therapies

**Table 2** shows the association of ESR1 mutation status with clinicopathological characteristics at study entry.

We found a statistically significant association between prior exposure to AI (considering both the early and the advanced setting and the advanced setting alone) and the presence of ESR1 mutation (p = 0.010 and p = 0.008 respectively). Prior exposure to fulvestrant and everolimus for aBC was also associated with higher rate of ESR1 mutation (p = 0.004 and p = 0.022 respectively). However, when we performed logistic regression multivariable analysis, none of these factors remained statistically significant after adjusting for the other variables (prior exposure to chemotherapy, aromatase inhibitors, tamoxifen, fulvestrant, CDK4/6 inhibitors, everolimus) (data not shown). The majority of the patients (n = 35, 81%) had received two or less previous treatment for BC, with no differences in terms of ESR1 mutational status. Features related with disease burden were not associated with presence of ESR1 mutation. Notably, we found a numerically higher rate of ESR1 mutation in the case of visceral disease (eight out nine mutated patients, p = 0.123). LDH value as assessed at the time of liquid biopsy was available for 24 patients. All of the three patients with ESR1 mutation had high LDH, although this association was not statistically significant (p = 0.091).

## Survival Analysis

Median follow-up was 14.5 months (95%CI 12.0–17.0 months).

As shown in **Figure 3**, there was no significant difference in PFS between ESR1 wild-type and ESR1-mutated patients: median PFS was 13.6 months (95%CI 9.6–17.5 months) in ESR1 wild type population *versus* 6.4 months (95%CI 0.00–15.6 months) in ESR1 mutant patients (log-rank p = 0.283, HR 1.62, 95%CI 0.7–4.0, p = 0.288).

# DISCUSSION

During the natural history of HR+/HER2-negative mBC, the onset of endocrine resistance is the rule, and a deep understanding of underlying mechanisms remains an unmet medical need.

Analysis of tumor tissue allows obtaining crucial predictive and prognostic information to guide clinicians, although, due to its static nature, a tumor biopsy is not able to capture intra-tumor heterogeneity and temporal evolution under exposure to specific treatments. Furthermore, multiple-biopsy testing could affect patients' quality of life (QoL). In this perspective, liquid biopsy offers a charming tool to overcome these limitations.

In our work, we prospectively examined ESR1 status in the tissue of 43 patients with HR+/HER2- mBC and in matched plasma samples. Overall, our total mutation rate was 21%, consistent with main literature data (12, 14, 15, 26, 29).

In this study, the total concordance rate between ESR1 status on tumor tissue DNA and ctDNA was 91%. Among the five discordant cases described in our cohort, two out of six mutations detected in tumor tissue DNA were undetectable in ctDNA. A possible reason may be represented by the low concentration of cfDNA in these two patients. The other three discordant cases showed a mutation on ctDNA but not on matched tumor tissue DNA. This finding suggests that ctDNA might be able to represent the heterogeneity of mBC, particularly in the case of patients with multi-metastatic disease.

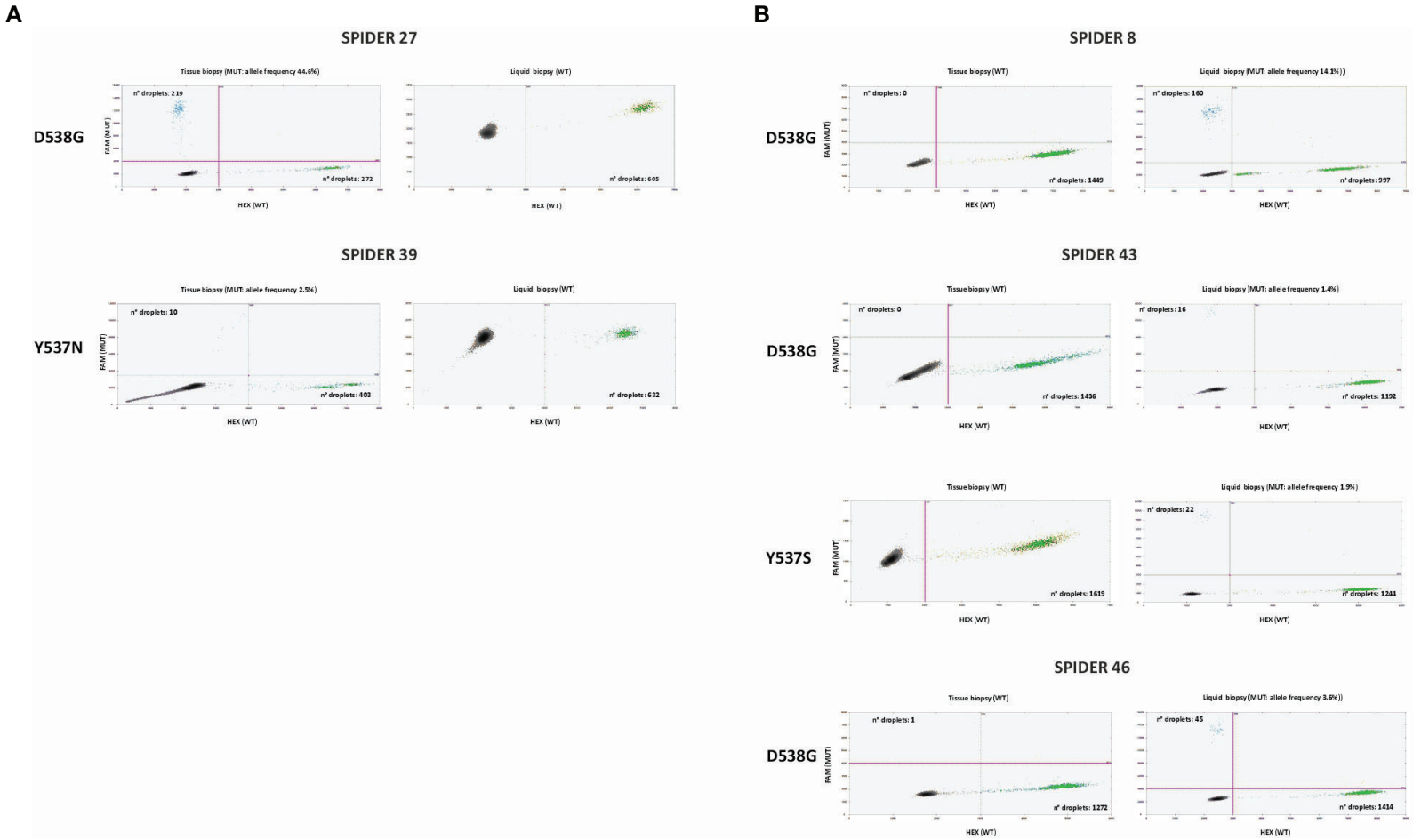


**FIGURE 1** | Discordant cases in ddPCR between tissue and liquid biopsy: ddPCR plots show the number of positive droplets for the indicated hotspot mutations (FAM+: blue dots), and the number of wt droplets (HEX+: green dots). Double positive droplets (FAM+/HEX+) were excluded from the analysis. **(A)** Samples mutated in tissue biopsy and WT in liquid biopsy. **(B)** Samples WT in tissue biopsy and mutated in liquid biopsy.


| ID | Tissue Biopsy | | Liquid Biopsy | |
|---|---|---|---|---|
| | *ESR1 mut* | % | *ESR1 mut* | % |
| **S8** | WT | / | D538G | 14.8 |
| **S10** | Y537S | 35.7 | Y537S | 1.1 |
| **S11** | E380Q | 84.9 | E380Q | 34.1 |
| **S15** | E380Q | 34.4 | E380Q | 2.1 |
| **S19** | D538G | 67.4 | D538G | 1.2 |
| **S27** | D538G | 43.1 | WT | / |
| **S39** | Y537N | 2.5 | WT | / |
| **S43** | WT | / | Y537S | 1.9 |
| | | | D538G | 1.4 |
| **S46** | WT | / | D538G | 3.7 |

**FIGURE 2** | ESR1 mutation in tissue and plasma samples. The lower panel shows the specific ESR1 mutations found and their representation in samples as percentage (%) of mutated allele.

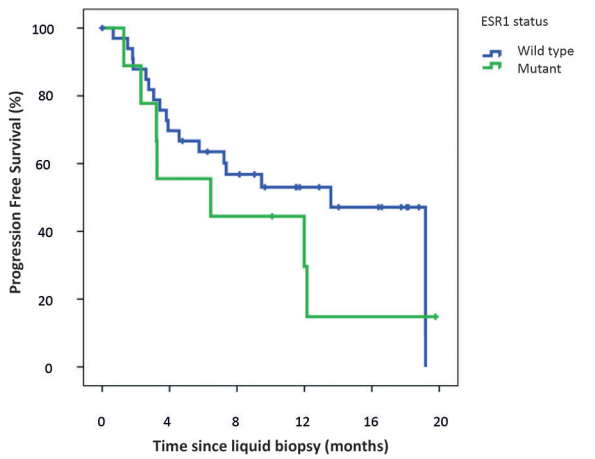


**FIGURE 3** | Kaplan–Meier progression-free survival analysis according to ESR1 status.

Available literature reports an overall concordance rate for ESR1 mutation between matched tissue and plasma samples ranging from 47 to 100%, although the majority of the data come from small series (**Table 3**) (15, 29, 30, 32, 36).

Altogether, our results are consistent with previous studies which evaluated ESR1 mutation by ddPCR and showed a rate of concordance of 74 to 97% (15, 29).

Across available data, concordance rates appear to be lower when the ESR1 mutation status on ctDNA is compared with sequencing results obtained from archival tumor tissue samples rather than recent tumor biopsies, as performed in our study.

All patients with a detectable *ESR1* mutation (in either tissue DNA or ctDNA) had a previous exposure to AI ($p = 0.010$), confirming the role of the selective pressure of hormonal-deprivation therapy in endocrine resistance development (17, 35). With regard to other therapies for advanced disease, a statistically significant association between ESR1 mutation and fulvestrant ($p = 0.004$) and everolimus ($p = 0.022$) was evident. It must be taken into account that all these patients received AI either previously (in case of fulvestrant) or concomitantly (in case of everolimus). Thus, the exposure to such therapies is a surrogate of prior treatment with aromatase inhibitor.

In a recent meta-analysis, the presence of ESR1 mutation was associated with worse PFS and OS in a population of patients with HR+/HER2-negative mBC (37).

In our population, the presence of ESR1 mutation did not impact on either PFS or OS, although this finding could be biased by the small sample size with limited follow-up. Moreover, the limited sample size did not allow conducting a survival analysis stratified by line of treatment.

Although this study has limitations, such as the small sample size, the mono-institutional enrollment, and ESR1 status assessment in a single laboratory, our results showed high concordance rate between tumor tissue and ctDNA (91%), providing evidence of reliability and feasibility of liquid biopsy to analyze ESR1 mutation in breast cancer patients. Moreover, the presence of ESR1 mutation in ctDNA of three patients lacking ESR1 mutations in the tissue suggests that liquid biopsy may capture the heterogeneous genetic landscape of metastatic tumors.

In conclusion, our data confirm the potential role of liquid biopsy as a valid and preferable non-invasive alternative to tissue biopsy for ESR1 mutation assessment in mBC patients. Moreover, it can also allow longitudinal tracking of ESR1

**TABLE 3 |** Studies investigating concordance of ESR1 mutation between tissue and plasma.

| Reference | Method | Total Mutation Rate | Overall concordance rate (%)* | Positive concordance rate (%)** |
|---|---|---|---|---|
| Schiavon G. (15) | ddPCR | 19/171 (11.1%)[§] | 30/31 (97%) | 3/4 (75%) |
| Chu D. (30) | NGS | 9/11 (82%) | 10/11 (91%) | 8/9 (89%) |
| | | 3/8 (37.5%)[§] | 2/5 (40%) | 0/5 (0%) |
| Yanagawa T. (32) | NGS | 11.3% | 5/5 (100%) | 0/5 (0%) |
| Sefrioui D. (33) | SANGER/ ddPCR | 6/7 (86%) | 5/7 (71.4%) | 4/6 (67%) |
| Takeshita T. (29) | ddPCR | 10/35(29%) | 26/35 (74%) | 1/10 (10%) |
| Lupini L. (34) | COLDPCR | 8/40 (20%) | 3/6 (50%) | 1/4 (25%) |
| Spoerke J. (35) | rtPCR/ BEAMing | 37.2%[§] | 22/47 (47%) | 11/36 (31%) |
| Wang P. (36) | ddPCR | 3/43(7%)[†] | 3/5 (60%) | 3/5 (60%) |
| | | 4/35 (11.4%) | | |

*Considering cases that were analyzed in both tissue and plasma samples and were both negative or both mutated.*

***Considering cases that were positive for ESR1 mutation in tissue DNA and/or ctDNA and were positive in both tissue and ctDNA.*

*[§]prospective cohort.*

*[†]primary tumor.*

*ddPCR, droplet digital polymerase chain reaction; NGS, next generation sequencing.*

mutations during the disease course at multiple timepoints without exposing patients to the risks related to invasive procedures. Clinical utility of this approach to guide treatment choices is currently under investigation.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IOV Ethics Committee (Cod. CESC IOV 2018/26, February 26, 2018). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

MVD conceived and designed the study. LU, JC, MLM, MF, and EZ performed molecular and pathology analyses. GV, GF, AM, EL, GG, CF, PC, VG, and MVD followed the patients. GV and LU acquired, analyzed, and interpretated data and drafted the work. MVD, VG, PC, and SI revised the work critically and approved the final version. All authors agree to be personally accountable for the content of the work. All authors contributed to the article and approved the submitted version.

## FUNDING

This research was supported by grant from Italian Ministry of Health (GR-2016-02361279 to MVD).

## ACKNOWLEDGMENTS

We thank Dr. Barbara Belletti, Division of Molecular Oncology, Centro di Riferimento Oncologico di Aviano (CRO), IRCCS, National Cancer Institute, 33081 Aviano, Italy and Dr. Ben Ho Park, M.D., Ph.D., Adjunct Professor of Oncology, Breast Cancer Program, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, for providing us with the pSEPT plasmid bearing ESR1 mutations. The QX200 ddPCR system (Bio-Rad Laboratories) was obtained through a grant provided by the University of Padova, Italy (2015).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.625636/full#supplementary-material

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**Conflict of Interest:** MVD reports personal fees for consultancy/advisory role from: Eli Lilly, Novartis, Celgene, Genomic Health, outside the submitted work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 18 November 2021
doi: 10.3389/fonc.2021.763790

# Circulating Tumor DNA and Minimal Residual Disease (MRD) in Solid Tumors: Current Horizons and Future Perspectives

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Circulating tumor DNA (ctDNA) is cell-free DNA (cfDNA) fragment in the bloodstream that originates from malignant tumors or circulating tumor cells. Recently, ctDNA has emerged as a promising non-invasive biomarker in clinical oncology. Analysis of ctDNA opens up new avenues for individualized cancer diagnosis and therapy in various types of tumors. Evidence suggests that minimum residual disease (MRD) is closely associated with disease recurrence, thus identifying specific genetic and molecular alterations as novel MRD detection targets using ctDNA has been a research focus. MRD is considered a promising prognostic marker to identify individuals at increased risk of recurrence and who may benefit from treatment. This review summarizes the current knowledge of ctDNA and MRD in solid tumors, focusing on the potential clinical applications and challenges. We describe the current state of ctDNA detection methods and the milestones of ctDNA development and discuss how ctDNA analysis may be an alternative for tissue biopsy. Additionally, we evaluate the clinical utility of ctDNA analysis in solid tumors, such as recurrence risk assessment, monitoring response, and resistance mechanism analysis. MRD detection aids in assessing treatment response, patient prognosis, and risk of recurrence. Moreover, this review highlights current advancements in utilizing ctDNA to monitor the MRD of solid tumors such as lung cancer, breast cancer, and colon cancer. Overall, the clinical application of ctDNA-based MRD detection can assist clinical decision-making and improve patient outcomes in malignant tumors.

**Keywords: circulating tumor DNA, minimum residual disease (MRD), biomarker, liquid biopsy, cancer**

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***Edited by:***
*Reza Safaralizadeh, University of Tabriz, Iran*

***Reviewed by:***
*Narges Dastmalchi, University of Tabriz, Iran*
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***Specialty section:***
*This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology*

***Received:*** *24 August 2021*
***Accepted:*** *03 November 2021*
***Published:*** *18 November 2021*

***Citation:***
*Peng Y, Mei W, Ma K and Zeng C (2021) Circulating Tumor DNA and Minimal Residual Disease (MRD) in Solid Tumors: Current Horizons and Future Perspectives. Front. Oncol. 11:763790. doi: 10.3389/fonc.2021.763790*

## INTRODUCTION

Liquid biopsy, defined as the analysis of cancer biomarkers in tumor-derived material extracted from cancer patients' bloodstream, urine, pleural effusion, cerebrospinal fluid, saliva, or bile, has recently gained growing attention in cancer diagnosis and treatment owing to its many benefits and application potential. Unlike traditional tissue biopsy, liquid biopsies are non-invasive, easily repeatable, and may offer a handy insight into tumor burden and treatment response.

Furthermore, the liquid biopsy may give a molecular snapshot of the primary tumor, minimizing bias in biopsy findings caused by sampling bias and intratumor heterogeneity. Nucleic acids, proteins, extracellular vesicles, and other biological components secreted into bodily fluids by cancer cells are among the analytes of liquid biopsies. Circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), circulating tumor RNA (ctRNA), exosomes, proteins, and metabolites as the analytes of liquid biopsies can be identified using biomarkers such as somatic point mutations, deletions, amplifications, gene fusions, DNA methylation markers, miRNAs, proteins, or metabolites. ctDNA is a potential biomarker since it contains tumor-specific genetic and epigenetic abnormalities and may be utilized in cancer diagnosis and prognosis prediction. The fact that symptoms of many cancer types are frequently absent at an early stage has resulted in extensive research efforts to create non-invasive, reliable, and cost-effective early detection techniques for these diseases. The bulk of the presently known research on the utilization of ctDNA is concerned with mutation detection. The study of ctDNA is addressed in the context of noninvasively detecting mutations that result in resistance mechanisms and monitoring treatment and disease response in cancer patients. Because the ctDNA percentages in total cell-free DNA (cfDNA) biofluid samples are extremely low, and their levels vary depending on the type and stage of cancer, highly sensitive assays are required to identify these tiny ctDNA fractions. Over the last several years, significant progress has been made in the development of ctDNA detection methods. PCR-based sequencing, which includes real-time quantitative PCR (qPCR), and digital PCR (dPCR) methods, is an alternative method for single-locus/multiplexed tests and targeted panels, while Next Generation Sequencing (NGS)-based sequencing, which includes Tagged-Amplicon deep sequencing (TAM-Seq), CAncer Personalized Profiling by deep sequencing (CAPP-Seq), and Duplex sequencing can be applied to panels of any size (1, 2). Notably, the revolution in ctDNA-based liquid biopsies has opened up new opportunities for cancer diagnosis, prognosis, monitoring, and treatment guidance (3).

Recent improvements in sequencing technology and ctDNA analysis have enabled non-invasive monitoring of the patient disease burden and assessment of molecular targets. In many tumor types, such as lung cancer, breast cancer, colon cancer, pancreatic cancer, and bladder cancer, ctDNA has been proven to be effective in detecting MRD (4). Patients with cancer may benefit from ctDNA testing to ascertain the presence of MRD and to forecast recurrence in the postoperative setting. For individuals undergoing adjuvant chemotherapy, the non-invasive and dynamic nature of the biomarker may potentially serve as a real-time indicator of adjuvant chemotherapy effectiveness. MRD tests may be utilized not only for early relapse detection and adjuvant therapy but also for initiating and monitoring systemic treatment, as well as drug resistance genotyping (5). Overall, MRD aids in the management of cancer at all stages, including screening, guiding adjuvant treatment, predicting relapse early, initiating systemic treatment and monitoring response, and genotyping resistance.

# CLINICAL UTILITY OF CIRCULATING TUMOR DNA (ctDNA)

## ctDNA Detection Methods

The amount of detectable ctDNA is determined by the tumor type, tumor load, and other biological processes such as plasma nuclease activity. cfDNA is fragmented DNA, with the overall quantity of ctDNA making up as low as 0.01% of the entire cfDNA. ctDNA-based NGS technology can identify not only somatic mutations but also copy number variation (CNVs) and structural rearrangement (6). Understanding the development of ctDNA detection technology is crucial to evaluating the clinical significance of ctDNA. When it comes to sensitivity and expense, there is always a compromise. Several techniques have been suggested to decrease the cost, errors, and background noise. Droplet digital polymerase chain reaction (ddPCR), beads, emulsion, amplification and magnetics (BEAMing), tagged-amplicon deep sequencing (TAm-Seq), cancer personalized profiling by deep sequencing (CAPP-Seq), whole-genome sequencing (WGS), and whole-exome sequencing (WES) are some of the most used ctDNA detection techniques (**Table 1**) (22, 23).

The ddPCR method distributes DNA samples into hundreds to millions of water-oil emulsion droplets. The advantages of ddPCR include its excellent sensitivity for identifying mutations and its low cost for absolute quantification. In comparison to NGS-based techniques, PCR-based methods have a much shorter turnaround time, with the majority of data being returned within 72 hours, as opposed to 1 to 2 weeks for massively parallel sequencing. The ddPCR method has the disadvantage of detecting only known variants and analyzing only a limited number of variants. ddPCR offers higher sensitivity than conventional quantitative PCR or NGS and a more straightforward workflow than alternative digital PCR methods like BEAMing. According to a meta-analysis, ddPCR has a high specificity (72.1%) and acceptable sensitivity (95.6%) for detecting EGFR mutations in cfDNA, which justifies its use in clinical practice as a supplement or conditional substitute for tissue biopsy for genotyping. It also appears to have a higher sensitivity than ARMS-PCR, especially in the early stages of lung cancer (24). Furthermore, KRAS G12/G13 mutations may be detected in a tiny quantity of unamplified cfDNA utilizing a droplet digital PCR multiplex technique, which has excellent agreement with conventional mutation testing for archival tumor tissue (25). Although ARMS, ddPCR, and BEAMing have excellent sensitivity and detection capabilities for various stages of cancer, their clinical applicability is restricted since these methods can only identify known mutations (23, 26, 27).

NGS is a high-throughput technique that can search for previously unidentified variations. As more therapeutically relevant molecular targets become available, NGS becomes more important in cancer. Although whole exome or whole genome sequencing may provide more detailed genomic information, ctDNA NGS techniques in clinical usage utilize hybrid capture panels or amplicon-based NGS to provide clinically relevant information with lower cost and higher sequencing depth. In the last decade, NGS has established itself

**TABLE 1** | Circulating tumor DNA (ctDNA) detection methods.

| Technique | Method | Advantages | Limitations | Reference |
|---|---|---|---|---|
| Allele-specific PCR | ARMS | Easy to set-up; Lowest cost | Low sensitivity; Detect specific genomic locations | (7) |
| Digital PCR | ddPCR | High sensitivity; Absolute quantification | Detect specific genomic locations; Limited in multiplexing | (8, 9) |
| | BEAMing | High sensitivity; Relatively inexpensive | Detect only known mutations | (10, 11) |
| Multiplex PCR-based NGS | TAm-Seq | High sensitivity; Lower cost than other NGS methods | Detect only known mutations; Less comprehensive than other NGS method | (12, 13) |
| | Safe-SeqS | High sensitivity; Lower cost than other NGS methods | Less comprehensive than other NGS method | (14, 15) |
| Hybrid capture-based NGS | CAPP-Seq | High sensitivity; Detects multiple mutation types; Broadly applicable without personalization; Lower cost than WGS/WES; Higher sequencing depth than WGS/WES | High cfDNA input; Detect only known mutations; Less comprehensive than WGS/WES | (16, 17) |
| | TEC-Seq | High sensitivity; Detects multiple mutation types; Broadly applicable without personalization; Lower cost than WGS/WES; Higher sequencing depth than WGS/WES | Less comprehensive than WGS/WES | (18) |
| Retrotransposon-based amplicon NGS | FAST-SeqS | Rapid aneuploidy assessment with lower cost than WGS/WES | Low sensitivity and specificity; Limited to aneuploidy detection | (19, 20) |
| Whole-genome sequencing (WGS) | WGS | The entire genome is interrogated; Broadly applicable without personalization | Limited sequencing depth; Low sensitivity; Expensive; Limited to SCNA | (21) |
| Whole-exome sequencing (WES) | WES | The entire exome is interrogated; Broadly applicable without personalization | Limited sequencing depth; Low sensitivity; Expensive | (21) |

*PCR, polymerase chain reaction; ddPCR, droplet digital polymerase chain reaction; BEAMing, bead, emulsion, amplification, and magnetics; TAm-Seq, tagged–amplicon deep sequencing; Safe-SeqS, safe-sequencing system; CAPP-Seq, cancer personalized profiling by deep sequencing; TEC-Seq, targeted error correction sequencing; FAST-SeqS, fast aneuploidy screening test-sequencing system; WGS, whole-genome sequencing; WES, whole-exome sequencing; SCNA, somatic copy number alteration; NGS, next-generation sequencing.*

as a reliable method for sequencing DNA and collecting genetic data. NGS works by analyzing millions of short DNA sequences in parallel, then aligning them to a reference genome or assembling them from a *de novo* sequence. Tagged-Amplicon deep sequencing (TAm-seq) and CAncer Personalized Profiling by deep sequencing (CAPP-Seq) are some of the techniques that are used to apply NGS to a target panel (23, 27). The enhanced TAm-Seq technique identified mutant alleles down to 0.02% allele fraction with 99.9997% per-base specificity. Samples with the optimum quantity of DNA had 94% mutations at 0.25% -0.33% allele fraction, compared to 90% mutations in samples with lower levels of input DNA (12). The integrated digital error suppression (iDES)-enhanced CAPP-Seq technique allowed biopsy-free profiling of EGFR kinase domain mutations with a sensitivity of 92% and a specificity of 96% (28). Overall, analytical sensitivity is limited by low levels of cfDNA in the blood and sequencing artifacts. More clinical investigation of novel approaches is required to overcome these constraints. Additionally, plasma cell-free DNA methylomes could allow for non-invasive, highly sensitive, low-cost, and accurate early tumor detection and classification. cfMeDIP-seq (cell-free DNA immunoprecipitation and high-throughput sequencing) for genome-wide bisulfite free plasma DNA methylation analysis is cost-effective based on its ability to enrich CPG-rich fragments that may provide additional information (29).

## Timeline of ctDNA Development

In 1948, cfDNA was identified in human blood plasma (**Figure 1**) (30). Leon et al. observed higher cfDNA levels in the serum of cancer patients in 1977 (31). Subsequent research revealed specific KRAS mutations in plasma DNA from pancreatic cancer patients in 1994 (32). Besides, circulating mutant DNA was utilized to monitor tumor dynamics in cancer patients undergoing surgery or chemotherapy in 2008 (33). A direct comparison of circulating tumor DNA with other circulating biomarkers (CA 15-3 and circulating tumor cells) and medical imaging revealed that ctDNA is an informative, specific, and highly sensitive metastatic breast cancer biomarker in 2013 (34). In 2015, detecting mutations in ctDNA was used to monitor MRD and predict the likelihood of early breast cancer recurrence, and customize adjuvant treatment strategies (35). In 2016, the United States Food and Drug Administration (FDA) approved the first "liquid biopsy test" (Cobas EGFR mutation Test V2) for patients with non-small cell lung cancer (NSCLC). In 2008, the FDA approved the first comprehensive liquid biopsy (Guardant 360 Assay) as an expedited access pathway device and the Cancer SEEK assay for cancer screening at an earlier stage as a breakthrough device. In 2019, FDA granted breakthrough device designation to Grail's multi-cancer blood test for the early detection of multiple cancer types. In 2020, FDA approved first liquid biopsy next-generation sequencing (NGS) companion diagnostic test (Guardant 360 CDx Assay) to detect specific types of the epidermal growth factor receptor (EGFR) gene mutations in patients with NSCLC. In 2021, FDA granted two breakthrough device designations to the Signatera test for molecular residual disease (MRD) assessment and recurrence monitoring. Additionally, accumulating evidence demonstrates the usefulness of ctDNA in cancer diagnosis, prognosis, disease progression, and treatment response (36–38).

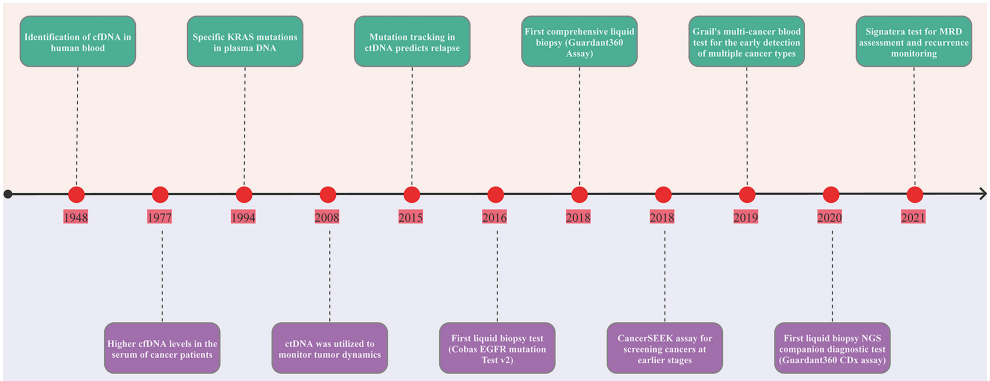


**FIGURE 1** | Timeline of the landmark in ctDNA analysis. The figure exhibits a timeline of selected significant milestones in ctDNA as applied to solid tumors. FDA, The United States Food and Drug Administration; MRD, minimal residual disease; ctDNA, circulating tumor DNA.

## ctDNA Analysis May Be an Alternative for Tissue Biopsy

Diagnostic tools for monitoring the molecular evolution through noninvasive techniques such as liquid biopsy are becoming accessible and will be a valuable tool for further improving personalized treatment in cancer. Especially, detection of molecular alterations utilizing ctDNA may be a viable approach for patients who do not have access to a tissue specimen or a high-quality biopsy (39). In a single-center analysis of 323 non–small cell lung cancer patients, 229 had concurrent plasma and tissue NGS or were unable to complete tissue testing. Tissue sequencing identified targetable mutations in 47 individuals (20.5%), whereas plasma sequencing identified 82 (35.8%). Moreover, 85.7% of patients treated with plasma next-generation sequencing–indicated treatment obtained a complete or partial response or stable disease (40). In patients with advanced non-small-cell lung cancer, analysis of ctDNA in blood samples may be used as a surrogate form of tumor biopsy for detecting EGFR and KRAS mutation status (41).

The ctDNA test is adequate to detect all driver DNA changes present in matched metastatic tissue in metastatic castration-resistant prostate cancer (mCRPC) patients, indicating that DNA biomarkers to guide mCRPC patient treatment based on ctDNA alone are feasible. Significant actionable alterations such as PTEN or BRCA2 loss are found in matched metastatic CRPC tissue samples, will not be missed by a well-designed ctDNA test. The excellent agreement between ctDNA and metastatic tissue biopsies in mCRPC indicates that ctDNA tests may be utilized to prognostically and predictively stratify patients (42). DNA damage repair (DDR) gene alterations identified in prostate cancer metastatic tissue or ctDNA were concordant with primary prostate cancer when clonal hematopoiesis was ruled out in genetic association analysis. Concordance in DDR gene alterations across prostate cancer samples was up to 84% (43).

The plasmaMATCH trial exhibited a significant degree of concordance across ctDNA assays and high sensitivity for mutations detected in tissue sequencing, particularly in contemporaneous advanced breast cancer samples. Advanced breast cancer patients with uncommon, potentially targetable HER2 and AKT1 mutations in ctDNA showed clinically significant responses to the HER2 inhibitor neratinib and the AKT inhibitor capivasertib, respectively, consistent with prior tissue sequencing-directed studies. These results validate the use of ctDNA testing to screen advanced breast cancer patients for rare mutations and show its clinical value (44). In a recent prospective investigation of metastatic triple-negative breast cancer, blood was shown to be a quicker and less invasive approach for molecular evaluation than tissue (45). In metastatic triple-negative breast cancer (TNBC), ctDNA was used to characterize somatic copy number alterations (SCNAs). SCNA identification is an attractive alternative to somatic mutation targeting since most tumors have SCNAs that may be easily detected using low-coverage WGS (46, 47). By comparing the SCRUM-Japan GI-SCREEN and GOZILA trials, ctDNA genotyping reduced screening time (11 vs. 33 days, $P < 0.0001$) and increased trial enrollment rate (9.5 vs. 4.1%, $P < 0.0001$) without compromising trial outcomes compared to tissue genotyping in advanced gastrointestinal cancer (48). Overall, ctDNA analysis is gaining popularity as a novel method of tumor genotyping.

## Recurrence Risk Assessment Using ctDNA

Increasing evidence suggests that ctDNA may be used as a predictor of relapse risk (**Figure 2**). The presence of ctDNA in follow-up samples was linked to future recurrence in all major breast cancer subtypes, with ctDNA identified before relapse in 22 of 23 patients (95.7%) with extracranial distant metastatic relapse. TNBCs had the highest ctDNA levels upon diagnosis, suggesting rapid cell growth and turnover. Early identification of ctDNA before treatment raised the likelihood of recurrence in early-stage breast cancer (49). The phylogenetic ctDNA profiling is used for ctDNA-driven treatment research that monitors the subclonal nature of early-stage lung cancer recurrence and metastasis (50). Another study showed that nonmetastatic colorectal cancer patients with positive ctDNA had a

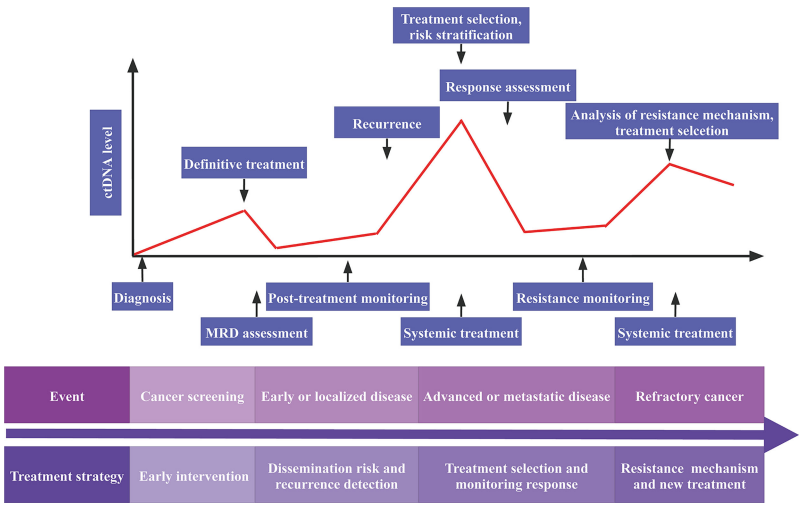


**FIGURE 2** | Potential clinical applications of ctDNA analysis. MRD, minimal residual disease; ctDNA, circulating tumor DNA.

recurrence incidence of 77%. Moreover, ctDNA positive patients had recurrence 3 months before radiologic or clinical evidence. With a median follow-up of 49 months, none of the 45 patients with negative ctDNA had a recurrence (51). The presence of ctDNA following cystectomy indicates the presence of residual cancer cells. After cystectomy, ctDNA was found in 17 individuals, 13 of whom had a recurrence. ctDNA-based recurrence detection outpaced radiographic imaging by up to a median of 96 days. Moreover, the dynamics of ctDNA throughout chemotherapy were related to disease recurrence (P =0.023) but not pathologic downstaging in ctDNA positive patients before or during therapy. The findings support that the feasibility of using ctDNA analysis for bladder cancer risk stratification, treatment monitoring, and early recurrence detection is feasible, and it offers a foundation for clinical trials evaluating early therapeutic approaches (52). Therefore, ctDNA may be utilized to identify early-stage cancer and predict recurrence in individuals with early-stage cancer.

## Monitoring Response Using ctDNA

There is mounting evidence that ctDNA analysis may be used to monitor the response to treatment intervention. After neoadjuvant chemotherapy, the presence of ctDNA was associated with a lower distant disease-free survival, disease-free survival, and overall survival in individuals with early-stage TNBC. Distant disease-free survival probability for ctDNA-positive individuals was 56% at 24 months, compared to 81% for ctDNA-negative patients (53). NCC-GP150 blood tumor mutational burden (bTMB) correlated well with WES matched tissue TMB (tTMB) (Spearman correlation = 0.62). A bTMB of 6 or higher was associated with improved progression-free survival and objective response rates in the anti-PD-1 and anti-PD-L1 therapy group, indicating that established NCC-GP150 with an optimized gene panel size and methodology was viable for bTMB estimation (54). Blood-based tumor mutational burden (bTMB) reliably identifies individuals who benefit from atezolizumab in second-line and higher NSCLC (55). 94% of patients with limited-stage (LS)-small-cell lung cancer (SCLC) and 100% of patients with extensive-stage (ES)-SCLC exhibited tumor-related alterations in their samples, including copy number alterations (CNAs) and somatic mutations. Targeted cfDNA sequencing reveals possible therapeutic targets in over 50% of SCLC patients using a simple cfDNA genomewide copy number method (56).

In advanced refractory CRC, patients with a plasma tumor mutation burden of 28 or more variations per megabase exhibited improved overall survival. Moreover, tumor mutation burden may be used to predict individuals with advanced refractory CRC who may benefit from durvalumab and tremelimumab (57). In the VIKTORY umbrella trail, ctDNA analysis revealed a strong link between high MET copy number and savolitinib response in patients with metastatic gastric cancer (58). At six weeks after immunotherapy, alterations in ctDNA levels suggested immunotherapeutic response and progression-free survival, and lower ctDNA levels were linked to better results. The results provide clues to the molecular characteristics associated with response to pembrolizumab in patients with metastatic gastric cancer (59). BRAF V600-mutant ctDNA identified in pre-treatment and on-treatment melanoma samples may be utilized as an independent indicator of clinical outcome in patients receiving dabrafenib or trametinib in combination with dabrafenib. In the COMbi-B cohort, the threshold of ctDNA was 64 copies per mL as a high or low risk of survival outcome, which was verified in the combi-B cohort. In the COMBI-d cohort, undetectable ctDNA at week four was associated with a prolonged progression-free and overall survival (60). Comprehensive ctDNA analysis reveals genetic variants that are clinically actionable in metastatic castration-resistant prostate cancer. Metastatic castration-resistant prostate cancer with TP53, BRCA2, or ATM mutations identified in plasma had substantially poorer

outcomes (61). The analysis of ctDNA from patients with carcinoma of unknown primary (CUP) demonstrates the potential of the ctDNA method to provide tailored treatments to CUP patients (62).

ctDNA analysis can identify residual proliferating disease in adjuvant settings and estimate tumor burden in metastatic settings and is a stratification indicator for immune-checkpoint inhibition. Moreover, ctDNA testing for immunotherapy predictors such as mutations, tumor mutational burden, and microsatellite instability provides a noninvasive alternative to tumor biopsy sampling. Quantitative changes in ctDNA levels early in the disease course have also been shown to be a valuable technique for assessing immune-checkpoint inhibition response that may supplement conventional imaging approaches (63). In an analysis of immune checkpoint inhibition across a broad range of cancer types, elevated pretreatment variant allele frequencies (VAF) were linked to worse overall survival, implying that VAF plays a prognostic role in patient outcomes. On-treatment VAF decreases and decreased on-treatment VAF were related to prolonged progression-free survival and overall survival, indicating that on-treatment ctDNA dynamics are predictive of immune checkpoint blockade benefit. Moreover, the combination of pretreatment and on-treatment VAF using ctDNA can identify long-term responders and adjudicated benefit among individuals with initial radiologically stable disease in advanced cancers (64).

Taken together, ctDNA may aid in the precise treatment of cancer and may help monitor patients' responses to treatment both during and after treatment.

## Resistance Mechanism Analysis Using ctDNA

ctDNA analysis can deepen the understanding of the mechanisms of drug resistance and provide more opportunities for precision medication for patients. The BENEFIT trial revealed that detecting EGFR mutations in ctDNA was an excellent method for identifying individuals who might benefit from gefitinib, and investigations of dynamic EGFR mutations and associated gene aberrances could help predict gefitinib resistance (65). BRCA reversion mutations are identified in 13% of platinum-resistant and 18% of platinum-refractory high-grade ovarian carcinoma pretreatment cfDNA and are associated with reduced therapeutic efficacy of rucaparib therapy. Besides, ctDNA analysis may identify several BRCA reversion mutations, indicating multiclonal heterogeneity in high-grade ovarian carcinoma (66). In almost all patients with mCRPC, clinically relevant genomic profiling of cfDNA was available, and it may offer significant insights on enzalutamide response and resistance (67). RAS and BRAF wild-type metastatic colorectal cancer patients responded to rechallenge with cetuximab and irinotecan in this phase 2 single-arm study. Only patients with RAS and BRAF wild-type ctDNA might benefit from the rechallenge, according to preplanned ctDNA profiling (68). CAPP-Seq analysis of ctDNA revealed that EGFR T790M mutation, MET amplification and ERBB2 amplification may lead to resistance to first- or second-generation EGFR-TKIs in NSCLC patients (69). Additional uses of ctDNA testing are being explored, including early identification of immunotherapy resistance and analysis of resistance pathways (63). In general, ctDNA provides a view into emerging mechanisms of resistance to targeted therapy or immunotherapy.

# UTILIZATION OF ctDNA FOR MINIMAL RESIDUAL DISEASE (MRD) DETECTION

MRD refers to residual tumor cells or biomarkers in the body after local or systemic cancer treatment, and its activation promotes tumor metastasis and recurrence, which is described as minimal residual disease, measurable residual disease, and molecular residual disease. Because the number of remaining cancer cells is likely to be so tiny that they may not cause any signs or symptoms, and they may even be undetectable by conventional techniques. The commonly used MRD detection techniques include qPCR (quantitative PCR), ddPCR (digital PCR), NGS (next-generation sequencing). Among them, NGS, as an emerging MRD detection technique, is gaining increasing attention and clinical application. Early detection of tumor metastasis and recurrence is critical for extending survival because smaller tumors have a better prognosis. MRD is a significant prognostic indicator that may help predict recurrence. Recently, the use of ctDNA analysis to identify MRD in solid tumors after curative-intent therapy and before clinical or radiographic disease recurrence has demonstrated significant therapeutic promise (**Figure 3**). Besides, MRD identification by ctDNA analysis was associated with a poor prognosis in patients with a malignant tumor. In this study, we describe the significance of ctDNA analysis for guiding adjuvant therapy in lung, breast, and colon cancers.

## Lung Cancer

The TRACERx study showed that over 99% of MRD-negative patients did not relapse and that MRD predicted relapse before conventional imaging. The time gap between the rise in ctDNA levels after surgery and the clinical diagnosis of cancer recurrence offers an opportunity for clinical intervention (50). The DYNAMIC study is the first prospective research on exploring ctDNA dynamic alterations in primary lung cancer patients after surgery. After tumor excision, ctDNA decays quickly in individuals with surgical lung cancer. Three days following surgery may be used as a baseline for lung cancer postoperative monitoring and may help guide clinical decisions (70). Moreover, the DYNAMIC study discovered that the half-life of ctDNA in individuals with radical resected lung cancer was only 35 minutes and that detecting MRD on the third day following R0 resection may be utilized as a baseline for postoperative lung cancer monitoring (70, 71).

A retrospective study demonstrates that ctDNA analysis can reliably detect posttreatment MRD in patients with stage I-III lung cancer, detecting residual or recurrent disease earlier than standard radiologic imaging, and making tailored adjuvant therapy more accessible to patients at an early stage with the

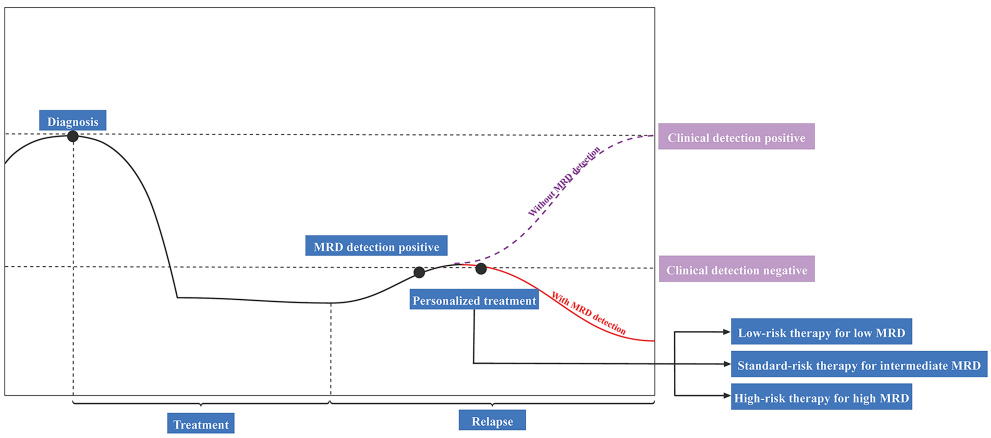


**FIGURE 3** | Potential clinical applications of ctDNA-based detection for minimal residual disease (MRD). ctDNA, circulating tumor DNA.

lowest disease burden. Freedom from progression (FFP) at 36 months after the MRD landmark was 0% in localized lung cancer patients with detectable ctDNA MRD and 93% in those with undetectable ctDNA MRD. The rate of MRD identification with single-mutation monitoring was 58%, considerably lower than the rate of 94% when all known variants were analyzed using cancer personalized profiling by deep sequencing (CAPP-seq), implying that monitoring multiple variants may improve MRD detection sensitivity in lung cancer (72). The assessment of MRD by ctDNA analysis predicts recurrence in early-stage lung cancer with excellent accuracy following therapy (72, 73). In another study that assessed the MRD in lung cancer patients using circulating single-molecule amplification and resequencing technology (cSMART), ctDNA status before surgery was a significant clinicopathological predictor for RFS and OS. ctDNA positive before surgery was associated with a 3.4- or 4.0-fold increased risk of recurrence or mortality, respectively. After surgery, the recurrence rate of ctDNA-positive patients was 63.3% (19/30). 89.5% of these patients who relapsed had detectable ctDNA within two weeks after surgery and were identified before imaging findings, with a median of 12.6 months (74).

In metastatic EGFR-mutant lung tumors, persistent EGFR-mutant ctDNA after six weeks of therapy was linked to early progression on osimertinib with bevacizumab and lower overall survival. In addition, persistently positive ctDNA may distinguish patients at risk for early progression of EGFR-TKIs and those who will benefit most from intensification therapy (75). In individuals who have had long-term responses to immune checkpoint inhibitors, ctDNA analysis can detect minimal residual disease and forecast the likelihood of disease progression. After a median of 26.7 months of immune checkpoint inhibitor therapy, ctDNA was tracked in 31 non–small cell lung cancer patients. 27 patients had undetectable ctDNA at the surveillance timepoint, and 93% (25/27) of them had not progressed. Besides, all four individuals with detectable ctDNA experienced disease progression. ctDNA monitoring may help to advance precision immunotherapy and provide more opportunities for early intervention in patients at high risk of disease progression (76).

Pre-treatment ctDNA and peripheral CD8 T levels are associated with durable clinical benefits from immune checkpoint inhibitors. ctDNA dynamics after a single infusion may help identify individuals who will obtain clinical benefits. Combining ctDNA with circulating immune cell profiling may determine patients who will benefit from treatment, and offer accurate, noninvasive, and early prediction of outcomes for NSCLC patients receiving immune checkpoint inhibitors (77). In the prospective INSPIRE study, all 12 patients whose ctDNA was cleared during pembrolizumab treatment had favorable clinical outcomes. With a median follow-up of 25.4 months after initial clearance, these 12 patients had sustained objective responses and 100% overall survival with a median of 25 months follow-up. For most patients with more than two ctDNA detections during ctDNA monitoring, increases in above-baseline ctDNA levels were linked to disease progression and poorer survival, with a median overall survival of 13.7 months. Besides, below-baseline ctDNA levels were associated with extended survival, with a median overall survival of 23.8 months. These findings indicated the therapeutic use of ctDNA-based monitoring in patients treated with immune checkpoint inhibitors (78).

## Breast Cancer

In a cohort of 55 early breast cancer patients undergoing neoadjuvant chemotherapy, identification of ctDNA following completing curative therapy accurately predicted metastatic recurrence. Mutation monitoring in serial samples increased sensitivity for recurrence prediction, with a median lead time of 7.9 months over clinical recurrence. Additionally, targeted capture sequencing of ctDNA could detect MRD-associated genetic events, and MRD detection more accurately predicted the genetic events associated with subsequent metastatic recurrence than primary cancer sequencing. Thus, mutation monitoring may be used to identify individuals with early-stage breast cancer who have an increased risk of recurrence.

Subsequent adjuvant therapy may target genetic events identified in the MRD, partially overcoming the barrier posed by intratumor genetic heterogeneity (35).

Another clinical study revealed that identifying ctDNA at diagnosis, prior treatment in early-stage breast cancer was linked to relapse-free survival. When compared to clinical recurrence, ctDNA detection had a median lead time of 10.7 months and was associated with recurrence in all breast cancer subtypes, suggesting that molecular relapse detection may be used to guide adjuvant treatment (79). A recent study demonstrated that personalized ctDNA analysis utilizing targeted digital sequencing (TARDIS) could identify residual disease in stage I-III breast cancer patients with excellent accuracy after neoadjuvant treatment. TARDIS identified ctDNA in all patients with 0.11% median variant allele frequency (VAF) before therapy. Following neoadjuvant treatment, ctDNA levels were significantly lower in patients who achieved pathological complete response (pathCR) than in patients with residual disease. Additionally, individuals with pathCR had a substantial decrease in ctDNA levels after neoadjuvant treatment. These results indicate that it is possible to accurately evaluate the molecular response and residual disease utilizing ctDNA analysis during neoadjuvant treatment (80). In addition, a novel, ultrasensitive assay was established to monitor numbers of specific tumor mutations to identify MRD following therapy. Whole-exome sequencing was performed to identify mutations in tumor tissue. Subsequently, an individualized MRD assay was used to detect mutations in the cfDNA. This approach allows the accurate detection of MRD at tumor fractions up to 100-fold lower than the genomic equivalent (GE) limit. The presence of MRD at one year was significantly associated with distant recurrence. The median lead time between the initial positive sample and recurrence was 18.9 months (81).

Pretreatment biopsies were sequenced to evaluate the role of MRD in neoadjuvant therapy for breast cancer using a massive parallel sequencing (MPS) panel, which enabled the detection of mutations and their investigation in plasma using droplet digital PCR (ddPCR) and tagged targeted deep sequencing (tTDS) as complementary approaches. Over one deleterious mutation was identified using tTDS in all four relapsed patients, with an average lead time of six months before clinical recurrence. However, just one relapsed patient could be detected using ddPCR. The results indicated that tTDS is a non-invasive tool for MRD detection in breast cancer patients (82).

In the neoadjuvant I-SPY study, serial ctDNA analysis can be utilized to determine pathologic complete response and metastatic recurrence risk in neoadjuvant-treated breast cancer. Patients who sustained ctDNA positivity at three weeks after initiation of paclitaxel had a substantially greater likelihood of developing residual disease after neoadjuvant chemotherapy (83% non-pathologic complete response) than those who cleared ctDNA (52% non-pathologic complete response). Following neoadjuvant chemotherapy, 100% of patients (N=17) who achieved pathologic complete response were ctDNA negative. For those who failed to achieve pathologic complete response (N=43), 14% of patients with ctDNA positivity exhibited a substantially higher risk of metastatic recurrence. 86% of patients failed to achieve pathologic complete response and tested negative for ctDNA had a favorable prognosis. Insufficient ctDNA clearance was a strong predictor of worse response and metastatic recurrence. ctDNA clearance was linked to better survival even in individuals who failed to achieve pathologic complete response. Personalized ctDNA monitoring during high-risk early breast cancer neoadjuvant chemotherapy may help assess therapy response and survival (83).

Early ctDNA dynamics revealed a strong relationship between on-treatment ctDNA and shorter progression-free survival in PIK3CA mutant breast cancer treated with palbociclib, taselisib, and fulvestrant. During triplet therapy, sequencing of longitudinal plasma ctDNA revealed evidence of genetic evolution (84). The PALOMA-3 trial showed that the change in PIK3CA ctDNA levels after 15 days of palbociclib and fulvestrant therapy significantly predict progression-free survival. These findings indicated that early ctDNA dynamics might serve as a reliable biomarker for CDK4/6 inhibitors, with early ctDNA dynamics showing diverse responses to treatment of tumor subclones (85).

## Colorectal Cancer

In a prospective study, ctDNA-positive stage I to III CRC patients exhibited a seven-fold increased risk of recurrence at postoperative day thirty. Relapse was seventeen times more probable in ctDNA-positive individuals following adjuvant chemotherapy. After adjuvant chemotherapy, all seven ctDNA positive patients relapsed. According to post-treatment monitoring, adjuvant chemotherapy eliminated 30% of ctDNA-positive patients. ctDNA-positive patients had 40 times the risk of disease recurrence than ctDNA-negative individuals during monitoring following definitive treatment. Serial ctDNA analysis showed disease recurrence 16.5 months before conventional radiologic imaging. Overall, ctDNA analysis may improve postoperative CRC treatment by risk assessment, adjuvant chemotherapy monitoring, and early recurrence identification (86). Positive postoperative ctDNA results had poor outcomes despite adjuvant treatment, with a three-year recurrence-free interval of 47% vs. 76% in patients with negative postoperative ctDNA, indicating that ctDNA analysis might serve as a prognostic biomarker for recurrence risk and adjuvant therapy benefit in stage III colon cancer (87).

Customized next-generation sequencing (NGS) panels were used to detect mutations in localized colon cancer tissues. In addition, ddPCR was used to monitor a series of plasma samples for known and high-frequency ctDNA mutations. Identifying ctDNA in serial plasma samples was linked to worse disease-free survival (DFS). The capacity to detect MRD improved to 87.5% by monitoring more than two variants in plasma. The presence of ctDNA following treatment was linked to early recurrence in individuals who received adjuvant chemotherapy. ctDNA could be detected at follow-up before radiological relapse, with a median lead time of 11.5 months, indicating that tracking ctDNA mutations may aid in identifying recurrence and that

identifying mutations in ctDNA occurring during or following adjuvant chemotherapy may aid in the detection of treatment resistance (88). ctDNA analysis after stage II colon cancer resection may demonstrate the presence of MRD, identify individuals at high risk of recurrence, and guide adjuvant treatment decisions. In a prospective cohort of 230 individuals with stage II colon cancer, parallel sequencing was performed to assess the capacity of ctDNA to identify MRD in 1046 plasma samples. Postoperative ctDNA was identified in 7.9% (14/178) of patients not treated with adjuvant chemotherapy. 78.6% (11/14) of these patients had radiologic recurrence at a median follow-up of 27 months. Only 9.8% (16/164) of the individuals with negative ctDNA had disease recurrence. The presence of ctDNA after completion of chemotherapy was linked to an inferior worse recurrence-free survival in those who underwent chemotherapy (89).

Recently, a plasma-only ctDNA assay that integrates genomic and epigenomic cancer signatures has been developed for tumor-uninformed MRD detection in postoperative colorectal cancer patients. Following completion of definitive treatment, 24% (17/70) of patients retained detectable ctDNA, and 88% (15/17) of these patients recurred. 24% (12/49) of the patients lacking detectable landmark ctDNA recurred. Sensitivity and specificity for landmark recurrence were 55.6% and 100%, respectively. The integration of longitudinal and surveillance analyses improved sensitivity to 69% and 91%, respectively. In comparison to genomic alterations alone, the integration of epigenomic signatures improved sensitivity by 25%–36%. The combination of epigenomic and genomic analyses improved sensitivity, indicating that plasma-only ctDNA MRD detection may be promising in clinical settings (90).

271 serial plasma samples were dynamically monitored with ctDNA during colorectal cancer patients with liver metastasis (CRLM) treatment to assess the impact of ctDNA on the prediction of adjuvant chemotherapy. Patients with a higher VAF level at their baseline ctDNA had a higher tumor burden, and reduced ctDNA levels during preoperative chemotherapy were associated with improved tumor response. The presence of ctDNA in patients after surgery and adjuvant chemotherapy was linked to a decreased recurrence-free survival (RFS). Patients with detectable ctDNA recurred after CRLM resection at a higher rate (79.4% vs. 41.7%) than those with undetectable ctDNA. Besides, recurrence rates were 77.3% for patients with detectable ctDNA following adjuvant chemotherapy and 40.7% for those with undetectable ctDNA. Patients with reduced ctDNA VAF had a 63.6% recurrence rate during adjuvant chemotherapy, compared to 92.3% for patients with elevated ctDNA VAF, indicating that dynamic ctDNA analysis in a post-adjuvant chemotherapy setting might be utilized to identify not only MRD but also to select the most appropriate individualized adjuvant treatment after CRLM resection (91).

## OTHER TUMORS

Monitoring the copy number status of HER2 in ctDNA is beneficial for the therapeutic effect of patients with HER2-positive gastric cancer and identifying treatment options for patients whose HER2 status changes to positive following recurrence. Plasma samples collected during postoperative follow-up periods indicated that high plasma HER2 ratios were observed at recurrence in seven of the thirteen patients who were diagnosed as HER2-negative (92). MRD identified by ctDNA distinguished stage I-III gastric cancer individuals at high risk of postoperative relapse and enabled new adjuvant therapy studies to prolong survival in adjuvant treatment settings. In a prospective cohort study, all patients who had ctDNA detected immediately after surgery eventually experienced a relapse. Positive ctDNA at any timepoint during longitudinal postoperative follow-up was associated with worse disease-free survival and overall survival, with a median time of 6 months before radiographic recurrence (93).

After applying the white blood cells-filtering approach, the presence of ctDNA in the CRITICS trial predicts recurrence when assessed within nine weeks following preoperative therapy and following surgery in individuals suitable for multimodal therapy. After a median follow-up of 42 months, all 11 resectable gastric cancer patients with no identifiable tumor-specific alterations at the postoperative timepoint were alive and recurrence-free. Of the nine patients who had detectable tumor-specific alterations at the postoperative period, six patients experienced disease recurrence and died of metastatic disease. In addition, patients with identifiable tumor-specific alterations had a substantially lower median event-free survival (18.7 months vs. not reached) and a 21.8-fold higher risk of recurrence as well as a considerably shorter median overall survival (28.7 months vs. not reached) after surgery. Moreover, the time to recurrence was determined by ctDNA analysis at 1.4 months, 8.9 months earlier than clinical detection (94).

The prognostic and predictive value of ctDNA was investigated using ultra-deep sequencing in patients with locally advanced bladder cancer before and after cystectomy, as well as during chemotherapy. Pre-chemotherapy ctDNA presence was strongly prognostic at diagnosis. For surveillance after cystectomy, ctDNA positivity accurately predicted all patients with metastatic recurrence with 100% sensitivity and 98% specificity. The dynamics of ctDNA throughout chemotherapy were associated with recurrence in individuals with ctDNA positivity before or during chemotherapy (52). In urothelial carcinoma patients who are positive for ctDNA and are at a high risk of recurrence, adjuvant atezolizumab may be associated with better outcomes than observation. At the initiation of treatment, ctDNA assay revealed 37% of patients were positive for ctDNA and had a dismal prognosis. The atezolizumab arm outperformed the observation arm in terms of disease-free survival and overall survival. At week 6, the atezolizumab arm (18%) had a greater rate of ctDNA clearance than the observation arm (4%) (95).

Comprehensive ctDNA alteration profiles offer a reliable strategy for evaluating tumor burden, with high consistency with imaging findings. It was able to detect the presence of tumors before imaging for an average of 4.6 months, and it is superior to serum biomarkers, such as alpha-fetoprotein, alpha-

fetoprotein-L3, and des-gamma-carboxy prothrombin. Moreover, it has the potential to accurately identify MRD in advance and forecast prognostic outcomes for relapse-free survival and overall survival. Comprehensive ctDNA alteration profiles may be used to evaluate prognostic risk and predict hepatocellular carcinoma occurrence (96).

## CHALLENGES AND FUTURE DIRECTIONS

An accurate understanding of the limitations of assay can effectively avoid making harmful decisions. Despite promising preliminary results, many obstacles exist to the widespread clinical application of ctDNA-based assay for treatment decision-making and tumor monitoring. In plasma, ctDNA levels tend to be variable and low, resulting in a variable detection threshold. In addition, negative ctDNA may be due to low copy number detection rather than the absence of ctDNA. The limited sensitivity of the ctDNA analysis is a critical challenge, particularly in patients with resected early-stage cancer, when plasma ctDNA levels are low. False negatives are inevitable due to the influence of biological variables such as mucinous histology, low DNA-shedding tumor, and hidden micrometastasis. NGS panels with a wide range of genomic or/and epigenetic alterations, larger sample volume, monitoring numerous mutations, serial testing, and fragment size analysis might enhance assay sensitivity. Besides, DNA fragments from the clonal hematopoiesis of indeterminate potential (CHIP) or non-neoplastic hematopoietic stem cells can cause false-positive ctDNA results, which can be reduced by utilizing advanced bioinformatics analysis or by comparing ctDNA sequencing with that of leukocytes and/or matched tumor tissues, but the optimal strategy is yet undetermined. A high-intensity cfDNA sequencing analysis method based on the combined analysis of cfDNA and white-blood-cell (WBC) gDNA enables *de novo* identification of tumor-derived alterations as well as interpretation of microsatellite instability, tumor mutational burden, mutational profiles, and the origins of somatic mutations found in cfDNA (97).

Integrating mutational information from peripheral blood cells (PBCs) is critical in liquid biopsy analysis to distinguish tumor-derived from clonal hematopoiesis (CH)-related mutations. Standard practice for NGS genomic analysis of cfDNA should include paired plasma-peripheral blood cell (PBC) sequencing to prevent findings from being misinterpreted (98). Another hurdle to overcome is the lack of uniformity among various ctDNA assays, which restricts the interpretation of presented results. The lack of standardization across ctDNA assays is another obstacle, which limits the understanding of available results. Discordance ctDNA findings are likely the consequence of several variables, including the time points of sample collection, sample collection process, storage procedure, library preparation process, unique molecular identifiers, variant calling, and targeted error correction. Normative methods for ctDNA collection, storage, and analysis are essential in ensuring the widespread utilization of ctDNA technology in regular clinical practice. Even though studies have provided convincing evidence supporting the role of ctDNA in the management of patients with resected early-stage cancer, these studies only included a small proportion of participants and lacked validation cohorts. Given the limited samples and observational findings, further large-scale randomized, controlled trials are required to verify and clarify the clinical usefulness of ctDNA in cancer.

The primary application of ctDNA assay in early-stage cancer treatment is its ability to identify MRD after primary tumor resection, thus enabling accurate risk assessment and adjuvant therapy. Adjuvant treatment may be avoided in the future for a significant proportion of ctDNA-negative individuals who are deemed high-risk. Moreover, ctDNA clearance may serve as an endpoint in adjuvant trials to assess the effectiveness of treatment, allowing for shorter follow-up times and smaller sample sizes. Besides, confirming adjuvant treatment duration based on ctDNA clearance will aid in reducing excessive toxicity. As a result, the ctDNA assay has enormous promise for speeding up the development of adjuvant therapies. Additional prospective studies will be conducted to evaluate the performance of the MRD assay. ctDNA surveillance during adjuvant therapy may aid in understanding the mechanisms of drug response and resistance, providing an opportunity for genome-based therapy before rapid disease progression. Neoadjuvant therapy is a rapidly developing approach for treating patients with early-stage cancer, and ctDNA may be an invaluable tool for monitoring tumor response in the neoadjuvant setting. It should be emphasized that excluding adjuvant treatment based on negative ctDNA assay is not appropriate, owing to the low degree of standardization of ctDNA testing procedures and the limitations of ctDNA testing technology.

Tumor-specific DNA methylation in plasma may be promising in cancer diagnosis, prognosis, and monitoring. With advances in molecular biology, detection technology, statistics, and machine learning, ctDNA methylation detection will make significant progress. Integrating DNA methylation analysis with genomic mutation detection may increase the sensitivity of detection. Incorporating various factors such as protein biomarkers, mutation-based, and epigenetic will get an accurate result. Genome-wide cell-free DNA fragmentation varies between cancer patients and healthy people. Fragmentation profiles of cfDNA in cancer patients seem to be caused by nucleosomal DNA mixes from both cancer cells and blood. DNA evaluation of fragments for early interception (DELFI) is a novel method that can identify a significant number of abnormalities in cfDNA *via* genome-wide evaluation of fragmentation patterns. By integrating DELFI with the detection of cfDNA sequence alterations, the sensitivity of detection was significantly improved. Since the fragmentation patterns appear to correlate with nucleosomal patterns, DELFI may help identify the tumor-derived ctDNA, which can be enhanced further utilizing clinical features, methylation alterations, and other diagnostic methods. Additionally, DELFI needs just a small amount of genome

sequencing, implying that it has the potential to be widely applied to cancer screening and management (99).

A single-tube methylation-specific quantitative PCR method (mqMSP), using ten different methylation markers, was able to quantitatively assess plasma samples as low as 0.05% of tumor DNA. The mqMSP assay is a cost-efficient and easy-to-implement clinical monitoring method for colorectal cancer recurrence, which aids in patient management after surgery. 55% (N=20) of recurrence colon cancer had mqMSP positivity in the postoperative plasma samples, which was associated with worse recurrence-free survival. Among the 20 recurrence patients, 70% exhibited detectable ctDNA prior to recurrence, with a median lead time of 8.0 months earlier than radiologic imaging (100). The plasma cell-free DNA methylomes are a sensitive method to detect ctDNA in low-level input DNA. In a large number of plasma samples from a variety of tumor types, this method has shown its effectiveness in detecting and classifying using plasma cell-free DNA methylomes (29). Moreover, plasma cfDNA methylomes exhibit specific characteristics in detecting and discriminating common primary intracranial tumors, which share cell-of-origin lineages and are difficult to differentiate with standard-of-care imaging (101). Cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP–seq) is a sensitive detection method that may identify cancers in the early stages. It can be used to accurately classify patients at all stages of renal cell carcinoma in plasma (the area under the receiver operating characteristic curve is 0.99), as wells as to identify renal cell carcinoma patients with urine cell-free DNA (the area under the receiver operating characteristic curve is 0.86), indicating that the utilization of plasma and urine cell-free DNA methylomes for the detection of renal cell carcinoma has the potential to revolutionize clinical practice (102).

Integrating methylated DNA immunoprecipitation with next-generation sequencing (MeDIP-seq) yields high-quality methylomes with typical resolutions of 100 to 300 bp at costs similar to those of capture-based methods. Moreover, the whole process, from DNA extraction to production of the MeDIP-seq library, may take around 3-5 days (103). Combining genetic and epigenetic characteristics of cfDNA can be used to distinguish between lung cancer and benign lung injury (BLN) plasma, indicating the potential of the multi-omics blood-based assay for non-invasive lung cancer management (104). Besides, ctDNA assays may have the potential to offer critical information on genomic heterogeneity. Adjuvant therapy may be guided by actionable mutations in clones that may vary from the primary tumor owing to clonal evolution or/and tumor heterogeneity. Surprisingly, there is evidence to substantiate the utility of ctDNA analysis in cancers of unknown origin (CUP) patients. Currently, the most data for ctDNA analysis mainly come from lung cancer, breast cancer, and colorectal cancer studies. The use of ctDNA will expand to a variety of tumor types, such as prostate cancer, and bladder cancer. It is anticipated that ctDNA will become increasingly extensively utilized and develop into a powerful tool for cancer diagnosis and treatment.

## CONCLUSION

As more evidence accumulates, it is becoming clear that ctDNA can be used as a biomarker for MRD detection and that it has the potential to aid in treatment decision-making. The advancement of ultra-sensitive ctDNA tests has the potential to improve cancer therapy. Moreover, ctDNA-based MRD detection may become an indispensable part of diagnosis and treatment. Using ctDNA detection techniques to evaluate MRD after therapeutic surgery may radically alter the course of adjuvant therapy for non-metastatic cancer. Serial postoperative ctDNA analysis may provide more accurate risk stratification for recurrence in addition to pathological staging. Moreover, postoperative ctDNA analysis may be used to adjust the intensity and duration of adjuvant treatment depending on the ctDNA findings. ctDNA monitoring can predict the effectiveness of adjuvant treatment and enhance the efficiency of adjuvant therapy trials. More studies are required to validate the clinical effectiveness of ctDNA and further enhance the sensitivity of ctDNA analysis. ctDNA assay standards should be established to ensure the repeatability of the results. Overall, the application of ctDNA-based MRD analysis is of great benefit in providing clinical decision support and enhancing patient survival outcomes in the era of precision medicine.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

## FUNDING

This work was supported by the National Natural Science Foundation of China (81660755); and the Science and Technology Project of Shenzhen of China (JCYJ20170307160524377 and JCYJ20190808162605484).

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 22 February 2022
doi: 10.3389/fonc.2022.840843

# Circulating Tumor DNA Mutations in Progressive Gastrointestinal Stromal Tumors Identify Biomarkers of Treatment Resistance and Uncover Potential Therapeutic Strategies

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**Specialty section:**
This article was submitted to Cancer Molecular Targets and Therapeutics, a section of the journal Frontiers in Oncology

**Received:** 21 December 2021
**Accepted:** 28 January 2022
**Published:** 22 February 2022

**Citation:**
Ko TK, Lee E, Ng CC-Y, Yang VS, Farid M, Teh BT, Chan JY and Somasundaram N (2022) Circulating Tumor DNA Mutations in Progressive Gastrointestinal Stromal Tumors Identify Biomarkers of Treatment Resistance and Uncover Potential Therapeutic Strategies. Front. Oncol. 12:840843. doi: 10.3389/fonc.2022.840843

Liquid biopsy circulating tumor DNA (ctDNA)-based approaches may represent a non-invasive means for molecular interrogation of gastrointestinal stromal tumors (GISTs). We deployed a customized 29-gene Archer® LiquidPlex™ targeted panel on 64 plasma samples from 46 patients. The majority were known to harbor *KIT* mutations ($n$ = 41, 89.1%), while 3 were *PDGFRA* exon 18 D842V mutants and the rest ($n$ = 2) were wild type for *KIT* and *PDGFRA*. In terms of disease stage, 14 (30.4%) were localized GISTs that had undergone complete surgical resection while the rest ($n$ = 32) were metastatic. Among ten patients, including 7 on tyrosine kinase inhibitors, with evidence of disease progression at study inclusion, mutations in ctDNA were detected in 7 cases (70%). Known somatic mutations in *KIT* ($n$ = 5) or *PDGFRA* ($n$ = 1) in ctDNA were identified only among 6 of the 10 patients. These *KIT* mutants included duplication, indels, and single-nucleotide variants. The median mutant AF in ctDNA was 11.0% (range, 0.38%–45.0%). In patients with metastatic progressive *KIT*-mutant GIST, tumor burden was higher with detectable *KIT* ctDNA mutation than in those without (median, 5.97 cm vs. 2.40 cm, $p$ = 0.0195). None of the known tumor mutations were detected in ctDNA for localized cases ($n$ = 14) or metastatic cases without evidence of disease progression ($n$ = 22). In patients with serial samples along progression of disease, secondary acquired mutations, including a potentially actionable *PIK3CA* exon 9 c.1633G>A mutation, were detected. ctDNA mutations were not detectable when patients responded to a switch in TKI therapy. In conclusion, detection of GIST-related mutations in ctDNA using a customized targeted NGS panel represents an attractive non-invasive means to obtain clinically tractable information at the time of disease progression.

**Keywords: liquid biopsy, imatinib, non-invasive, KIT, ctDNA (circulating tumor DNA)**

## INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the commonest mesenchymal neoplasm originating from the gastrointestinal tract. GISTs are classically defined by activating oncogenic mutations in *KIT* (KIT proto-oncogene receptor tyrosine kinase) (80%) or *PDGFRA* (platelet-derived growth factor receptor alpha) (10%) genes (1–3). In advanced stages, most patients benefit from targeted therapy using the tyrosine kinase inhibitor (TKI) imatinib, though acquired resistance and disease progression associated with the development of secondary mutations usually occur after 18 to 24 months. Under pharmacological pressure, secondary mutations can develop, conferring resistance to imatinib. In imatinib-resistant GISTs, secondary mutations typically occur in the ATP-binding pocket (exon 13) or in the kinase activation loop (exon 17) (4).

In the contemporary management of patients with advanced GISTs, radiological response evaluation following TKI treatment remains standard of care, and there is currently no specific blood-based biomarker for the purposes of monitoring disease progression or to determine mechanisms responsible for acquired TKI resistance. At the time of disease progression, knowledge of the specific secondary mutations could enable tailoring of treatment, though this requires invasive tumor biopsy at the site of progression. Understandably, such an approach does not comprehensively capture the evolving global tumor landscape and may not yield viable results that reflect tumor heterogeneity (5). In addition to defining resistance pathways, the secondary mutations also have therapeutic implications. Previous preclinical studies have suggested that sunitinib is preferentially more active against mutations in the ATP-binding pocket, while regorafenib has increased activity against mutations in the kinase activation loop (6).

The optimal approach to determine mechanisms of acquired resistance during therapy and guide personalized approach to subsequent management remains to be elucidated. Non-invasive tumor mutation profiling using several liquid biopsy cell-free circulating tumor DNA (ctDNA)-based approaches has been explored in patients with GISTs (7). While some of these approaches have been shown to capture the molecular heterogeneity of the whole tumor, their utility is limited to patients with high tumor burden as a result of suboptimal assay sensitivity, and their practical use in the clinic remains in question. In this study, we investigated a liquid biopsy approach for the detection of primary and secondary acquired mutations in patients with GISTs, using a customized Archer® LiquidPlex™ targeted panel. The advantage of Archer® LiquidPlex™ includes the Anchored Multiplex PCR (AMP™) enrichment chemistry, in which ctDNA fragments are ligated to molecular barcodes that allow for error correction for confident variant reporting. Furthermore, Archer® LiquidPlex™ is able to capture a fragment size that is smaller than 160 base pairs, a size that can be missed by other platforms. Finally, Archer® LiquidPlex™ can detect variants at 0.3% allele frequency with ctDNA input that is as low as 1 ng.

## PATIENTS AND METHODS

### Study Cohort

Blood samples were collected from patients who were diagnosed with GISTs and seen at the National Cancer Centre Singapore between April 1998 and September 2021. A total of 64 plasma samples and 7 whole blood samples from 46 patients were included in the final analysis. Relevant demographical and clinical information were collected and utilized for the analysis. For all GISTs diagnosed at our center, Sanger sequencing was routinely performed to detect *KIT* and *PDGFRA* gene mutations. Selected cases may undergo panel testing *via* next-generation sequencing at the discretion of the managing physician. All data were obtained at the time of diagnosis or subsequent follow-up. Written informed consent for use of biospecimens and clinical data was obtained in accordance with the Declaration of Helsinki. The research study was carried out with approval from the SingHealth Centralised Institutional Review Board (CIRB 2018/3182). The datasets created and analysed during this study are available from the corresponding authors upon reasonable request.

### Extraction and Quantification of ctDNA from Plasma and Whole Blood

Whole blood samples were collected in 10-ml EDTA-coated tubes (BD, Cat. 368589) and processed within 2 h of collection using an in-house protocol. The samples were centrifuged for 10 min at 300 × *g* at room temperature to separate plasma from red blood cells. Plasma layer was harvested in 1-ml aliquots in 1.5-ml Microcentrifuge Tubes (Axygen, MCT-150-C) and spun again in a microcentrifuge at 9,720 × *g* at 4°C. Plasma was collected and stored at −80°C until use. QIAamp Circulating Nucleic Acid Kit (Catalog #55114, Qiagen) was used to isolate ctDNA from plasma by following the manufacturer's instruction. Purified ctDNA was stored at −20°C.

The following is the protocol for extracting ctDNA from frozen whole blood. DNA were isolated by using DNeasy Blood & Tissue Kit (Qiagen) with the following modification to the protocol. Briefly, proteinase K was added to whole blood at the following volume ratio (proteinase K:whole blood, 1:10). Subsequently, Buffer AL (with no ethanol added) was added into thawed blood, containing proteinase K, at a volume ratio of 6:1 before incubating at 56°C for at least 10 min. Absolute ethanol was added at a volume that was equal to that of Buffer AL used. The number of DNA purification columns used was dependent on the volume of whole blood. The ratio was 1 column per 200 µl of thawed whole blood. Subsequent steps followed the manufacturer's instruction.

Once eluted, DNA was obtained from whole blood, Mag-Bind® Total Pure NGS (Omega Bio-tek) paramagnetic beads were used to separate the genomic DNA from ctDNA; 0.6× bead volume (calculation was based on the volume of the DNA eluent) was added to the DNA eluent, and the beads were used to remove the genomic DNA. The beads containing the genomic DNA was isolated from the supernatant by a magnetic column. Subsequently, the leftover supernatant was moved to a clean microfuge tube where 2× bead volume (calculation was based on the volume of the original DNA eluent) was added. This volume

of beads would isolate out the ctDNA from the supernatant. The beads were isolated by magnet, washed, and ctDNA was eluted according to the manufacturer's instructions. The profile of the eluted ctDNA fraction was visualized on Agilent TapeStation by using Agilent genomic DNA ScreenTape (Agilent Technologies, CA, USA) to ensure that there was no contaminating genomic DNA. If genomic DNA was still present in the eluted ctDNA fraction, the DNA clean-up would be repeated. Purified ctDNA, from plasma or whole blood, were quantified by using Qubit dsDNA assay kit (Thermo Fisher Scientific). Purified ctDNA profile was also visualized on Agilent TapeStation by using Agilent High Sensitivity D100 ScreenTape.

## Next-Generation Sequencing Library Construction and Sequencing

NGS libraries were made from, depending on individual sample ctDNA yield, 5 ng to 37 ng (median: 10.1 ng) of ctDNA input. The NGS libraries were constructed by following the Archer® LiquidPlex™ Protocol for Illumina® (Invitae). We are using a customized Archer® LiquidPlex™ targeted panel, dubbed LiquidPlex NCCS GIST 18265 v1.0, that consists of probes that target specific exons of 29 genes that are known to associate with cancer and encompass known somatic mutations of GISTs (**Supplementary Table 1**). The libraries were paired-end sequenced (2 × 150 bp) for 5–20 million raw reads with at least 5% PhiX by using the standard Illumina NGS protocol on the NovaSeq platform (Novogene).

## Bioinformatics Analysis

The raw sequencing data were analyzed with Archer Analysis pipeline (version 6.2.7; https://archerdx.com/technology-platform/analysis) as previously reported (8). We used the default settings for detecting variants that were statistically significant [in our case, the variant must have an allele frequency (AF) outlier $p$-value < 0.05]. For ctDNA samples where no significant variants were detected by the default setting, we would manually check all the mapped NGS reads for the presence of *KIT*, *PDGFRA*, or other variants that were previously detected by Sanger sequencing of the corresponding tumor. If such variant was detected, it would only be reported as a significant variant if AF outlier $p$-value < 0.05.

## Statistical Analysis

Comparisons of tumor size with detectable ctDNA were performed using Mann–Whitney $U$ test. All statistical evaluations were made assuming a two-sided test with a significance level of 0.05 unless otherwise stated. All tests were performed using MedCalc statistical Software for Windows version 19.0.4 (MedCalc Software, Ostend, Belgium).

# RESULTS

## Patient Characteristics

A total of 46 patients were included in the study (**Supplementary Figure 1**). The median age at study enrolment was 63.7 years (range, 30.3 to 89.1 years). Twenty-six (56.5%) were male and 20 (43.5%) were female. Primary tumor locations were stomach ($n$ = 24, 52.2%), small bowel ($n$ = 19, 41.3%), and rectum ($n$ = 3, 6.5%). The majority were known to harbor *KIT* mutations ($n$ = 41, 89.1%), while 3 patients (NCCS-GIST-06, NCCS-GIST-43, and NCCS-GIST-44) were *PDGFRA* exon 18 D842V mutants and *KIT* wild type. Two cases (NCCS-GIST-45 and NCCS-GIST-46) were wild type for both *KIT* and *PDGFRA*, one of which harbored *KRAS* exon2 G12V mutation. In terms of disease stage, 14 (30.4%) were localized GISTs that had undergone complete surgical resection. The rest ($n$ = 32) were metastatic GISTs at various points of their treatment trajectories. Importantly, 10 patients, including 7 on TKI treatment, had evidence of disease progression at study inclusion and were the focus of the study. Clinical and demographic characteristics of all patients are summarized in **Table 1**.

## Molecular Profiling Using ctDNA

Blood samples of the 46 patients were drawn at study inclusion. Targeted exon panel sequencing was performed to identify mutations in plasma ctDNA. Among the 10 patients with metastatic GIST with evidence of disease progression, mutations in ctDNA were detected in 7 cases (70%). Known somatic mutations in *KIT* ($n$ = 5) or *PDGFRA* ($n$ = 1) in ctDNA were identified only among 6 of the 10 patients (**Figure 1**).

**TABLE 1** | Characteristics of GIST patients at enrolment.

| Characteristic | *N* (%) |
|---|---|
| *Total* | 46 (100) |
| *Age at inclusion (years)* | |
| Median (range) | 63.4 (30.3 to 89.1) |
| *Sex* | |
| Male | 26 (56.5) |
| Female | 20 (43.5) |
| *Primary tumor location* | |
| Stomach | 24 (52.2) |
| Small intestine | 19 (41.3) |
| Rectum | 3 (6.5) |
| *Disease stage* | |
| Localized | 14 (30.4) |
| Metastatic | 32 (69.6) |
| *Evidence of progression* | |
| No | 36 (78.3) |
| Yes | 10 (21.7) |
| *Known somatic mutations* | |
| KIT† | 41 (89.1) |
| PDGFRA (exon 18 D842V) | 3 (6.5) |
| KIT/PDGFR wild-type‡ | 2 (4.3) |
| *Adjuvant therapy (localized)** | |
| Imatinib | 5 (35.7) |
| None | 9 (64.3) |
| *Palliative therapy (metastatic)** | |
| Imatinib | 18 (56.3) |
| Sunitinib | 2 (6.3) |
| Pazopanib | 1 (3.1) |
| Avapritinib | 1 (3.1) |
| None | 10 (31.3) |

*†Exon 11 (n = 29), exons 11 and 13 (n = 1), exons 11 and 17 (n = 2), exon 9 (n = 5), site unknown (n = 4).*
*‡One case of KIT/PDGFRA wild type known to harbor KRAS Exon2, c.35G>T/p.Gly12Val.*
**Treatment at time of study inclusion.*

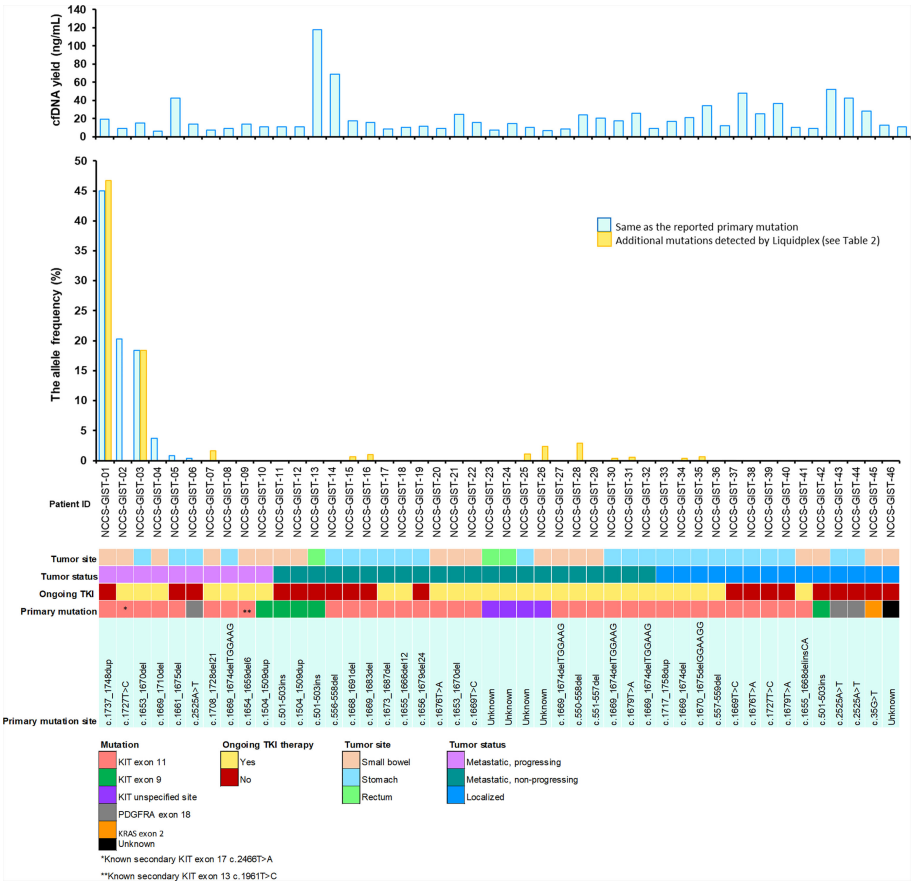


**FIGURE 1** | Overview of study cohort and plasma ctDNA mutation detection. Clinical and molecular characteristics of the 46 patients included in the study are shown. For the mutant allele frequency bar chart, the light blue bar indicates mutants that were detected in ctDNA are the same as that reported for the corresponding primary tumor. The gold-colored bar indicates additional mutants detected in ctDNA that is not reported in the corresponding primary tumor. See **Table 2** for more information on the mutation profiles of both the primary tumor and the corresponding ctDNA.

These *KIT* mutants included short sequence tandem duplication, indels, and single-nucleotide variants. The median mutant AF in ctDNA was 11.0% (range 0.38%–45.0%). In patients with metastatic progressive *KIT*-mutant GIST, tumor burden (as measured by the average diameter of the 3 largest lesions) was higher with detectable *KIT* ctDNA mutation than in those without (median, 5.97 cm vs. 2.40 cm, $p = 0.0195$) (**Figure 2**). The 4 cases with undetectable primary *KIT* mutations include GISTs with exon 11 c.1708_1728del (NCCS-GIST-07), exon 11 c.1669_1674del (NCCS-GIST-08), exon 11 c.1654_1659del and exon 13 c.1961T>C (NCCS-GIST-09), and exon 9 c.1504_1509dup (NCCS-GIST-10). None of the known tumor mutations were detected in ctDNA for localized cases ($n = 14$) or metastatic cases without evidence of disease progression ($n = 22$). In patient NCCS-GIST-02, only the known *KIT* exon 11 c.1727T>C mutation, but not the exon 17 c.2466T>A mutation was detected in ctDNA. On the other hand, in patient NCCS-GIST-03, ctDNA identified *KIT* exon 17 c.2467T>G on top of the known exon 11 c.1653_1670del mutation—this patient had prior imatinib-resistant GIST (no molecular evaluation done apart from time of diagnosis) and was progressing despite the 4th-line treatment with pazopanib. For patient NCCS-GIST-07 with small bowel GIST progressing on 1st-line treatment with imatinib, ctDNA detected *TP53* c.879_880del and *SETD2* c.2238del mutations, but not the known *KIT* exon 11 c.1708_1728del mutation (**Table 2**).

Mutations in *SETD2*, previously reported to confer worse prognosis in GIST (9), were detected in ctDNA in 3 patients with metastatic GIST. In patients with localized GIST following surgical resection ($n = 14$), including 5 on adjuvant imatinib, no known tumor mutations could be detected from ctDNA. Likewise, none could be detected from ctDNA from patients with metastatic GIST without evidence of disease progression ($n = 22$), including 15 patients on TKI treatment. In this group of patients, mutations in *IDH2* ($n = 3$), *KRAS* ($n = 3$), *KIT* ($n = 3$), and *SETD2* ($n = 1$) were identified at low AF (median, 0.86%; range, 0.68 to 2.9%), though the corresponding mutation in the primary tumor was not known.

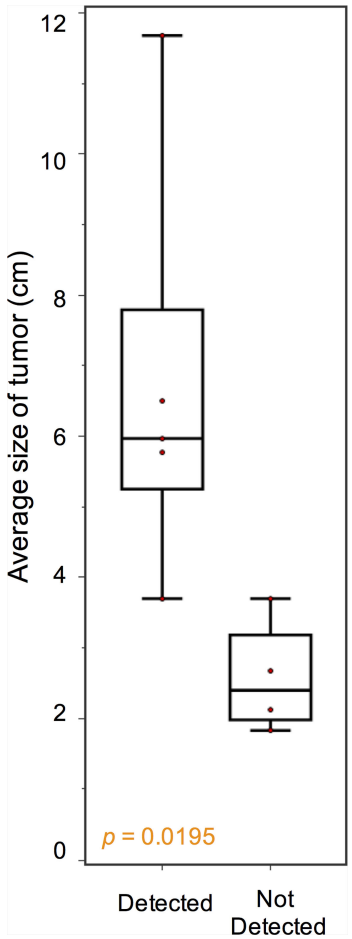


**FIGURE 2** | Association between *KIT* ctDNA mutation detection and tumor burden. In patients with advanced progressive GIST, tumor burden, as measured by the average diameter of the 3 largest lesions, was higher with detectable *KIT* ctDNA mutation than in those without (median, 5.97 cm vs. 2.40 cm, $p = 0.0195$).

## ctDNA Mutation Detection in Patients Progressing on Treatment

Serial plasma samples were available for 6 patients with metastatic GIST who underwent TKI therapy. For NCCS-GIST-17 (**Figure 3A**), the *KIT* exon 11 c.1673_1687del variant, as detected in the tumor, was not detected in the ctDNA from the first plasma sampling when no disease progression was evident (stable disease, SD) (week 0). In the ctDNA from the second plasma sampling at the time of disease progression in the primary stomach tumor (week 26), 2 *KIT* variants were detected, namely, exon 11 c.1673_1687del (AF = 3.1%) and exon 17 c.2485G>C (AF = 3.4%), the latter of which was a new variant not previously detected in the tumor. Additionally, a *PIK3CA* c.1633G>A variant was also detected (AF = 2.6%). Interestingly, in the ctDNA from the third plasma sampling (week 42, with progressive liver metastases), the *PIK3CA* c.1633G>A variant AF increased by nearly 32-fold (AF = 82.5%) while those *KIT* variants were reduced by at least 14.2-fold (exon 11 c.1673_1687del, AF = 0.15%; exon 17 c.2485G>C, AF = 0.24%). Generally, the detection of the *KIT* and *PIK3CA* variants in the ctDNA positively correlated with disease progression as evaluated by computed tomography (CT) scans.

For patients NCCS-GIST-04, NCCS-GIST-02, and NCCS-GIST-09, multiple plasma samplings at different time points (4–7 samples) have allowed for the monitoring of dynamic changes to the different *KIT* variants detected in the ctDNA in response to different types of TKI used. In NCCS-GIST-04 (**Figure 3B**) and NCCS-GIST-02 (**Figure 3C**), *KIT* variants detected in ctDNA progressively decreased and were not detected in ctDNA when patients were responsive to TKI treatments. However, we observed that after a period of positive drug response, known *KIT* variants re-emerged in ctDNA with additional new variants that were not previously detected in tumor. The emergence of additional new *KIT* variant correlated with resistance to corresponding TKI treatment and disease progression.

Patient NCCS-GIST-04 switched from imatinib to ripretinib (at week 0) at the time of disease progression based on imaging assessment (**Figure 3B**). Imaging at week 7 confirmed response to ripretinib therapy—correspondingly, the AF for the *KIT* exon 11 c.1669_1710del variant fell from 3.68% at week 0 to 0% at week 7. Subsequently from week 7 to week 19, no *KIT* variant was detected in the ctDNA, and this coincided with a state of continued disease stability for the patient. At week 25, disease progression was detected on CT scan, and this coincided with the re-emergence of the *KIT* variant exon 11 c.1669_1710del (AF = 1.1%) and the detection of a new *KIT* variant exon 17 c.2467T>G (AF = 0.59%). At week 43, the AF values for both *KIT* variants were still detectable, albeit reduced (exon 11 c.1669_1710del, AF = 0.16%; exon 17 c.2467T>G, AF = 0.18%). This coincided with the patient switching to sunitinib. Despite the reduction in AF values for the 2 *KIT* variants, the CT scan showed that the disease had progressed with increased tumor size.

On the other hand, NCCS-GIST-02 switched from avapritinib to imatinib and subsequently ripretinib as a result of lack of response in terms of tumor reduction in the first 8 weeks of monitoring; the *KIT* variants from exon 11 c.1727T>C and exon 17 c.2466T>A became undetectable from week 10 to week 16 (**Figure 3C**), correlating with positive response to ripretinib treatment. Subsequently, ctDNA collected from week 28 and week 33 contain not only the previously detected *KIT* variants from exon 11 c.1727T>C and exon 17 c.2466T>A, but also a new exon 13 c.1961T>C variant. This indicated the emergence of a novel TKI-resistant variant that correlated with disease progression. Conversely, for NCCS-GIST-09 (**Figure 3E**), despite disease progression, no *KIT* variant, as previously detected in tumor, was detected in ctDNA collected at the different time points. There was, however, a *KRAS* c.194G>A variant that was detected at week 41 albeit at low AF (0.84%), but it was not detected at the last sampling at week 48.

There were 2 patients with 2 serial samplings, namely, NCCS-GIST-03 and NCCS-GIST-08. For NCCS-GIST-03, 2 *KIT* variants were detected (**Figure 3D**). The exon 11 c.1653_1670del variant was previously detected in tumor, while

**TABLE 2 |** Mutational profiles of tumor and plasma ctDNA.

| Patient ID | Primary site | Disease status | Treatment at time of study inclusion | Known tumor mutation | Mutation in ctDNA | Allele frequency |
|---|---|---|---|---|---|---|
| NCCS-GIST-01 | Small bowel | Metastatic† | None | KIT Exon 11 c.1737_1748dup | KIT Exon 11 c.1737_1748dup | 45 |
| | | | | | SETD2 c.1844C>T | 46.7 |
| NCCS-GIST-02 | Small bowel | Metastatic† | Avapritinib | KIT Exon 11 c.1727T>C | KIT Exon 11 c.1727T>C | 20.3 |
| | | | | KIT Exon 17 c.2466T>A | | |
| NCCS-GIST-03 | Gastric | Metastatic† | Pazopanib | KIT Exon 11 c.1653_1670del | KIT Exon 11 c.1653_1670del | 18.4 |
| | | | | | KIT Exon 17 c.2467T>G | 20.3 |
| NCCS-GIST-04 | Small bowel | Metastatic† | Imatinib | KIT Exon 11 c.1669_1710del | KIT Exon 11 c.1669_1710del | 3.68 |
| NCCS-GIST-05 | Gastric | Metastatic† | None | Kit Exon 11 c.1661_1675del | Kit Exon 11 c.1661_1675del | 0.81 |
| NCCS-GIST-06 | Gastric | Metastatic† | None | PDGFRA Exon 18 c.2525A>T | PDGFRA Exon 18 c.2525A>T | 0.38 |
| NCCS-GIST-07 | Small bowel | Metastatic† | Imatinib | KIT Exon 11 c.1708_1728del | TP53 Exon 8 c.879_880del | 1.68 |
| | | | | | SETD2 c.2238del | 1.32 |
| NCCS-GIST-08 | Gastric | Metastatic† | Imatinib | KIT Exon 11 c.1669_1674del | | |
| NCCS-GIST-09 | Small bowel | Metastatic† | Sunitinib | KIT Exon 11 c.1654_1659del | | |
| | | | | KIT Exon 13 c.1961T>C | | |
| NCCS-GIST-10 | Small bowel | Metastatic† | Imatinib | KIT Exon 9 c.1504_1509dup | | |
| NCCS-GIST-11 | Small bowel | Metastatic | None | KIT Exon 9 c.501-503ins | | |
| NCCS-GIST-12 | Small bowel | Metastatic | None | KIT Exon 9 c.1504_1509dup | | |
| NCCS-GIST-13 | Rectum | Metastatic | None | KIT Exon 9 c.501-503ins | | |
| NCCS-GIST-14 | Gastric | Metastatic | None | KIT Exon 11 c.556-558del | | |
| NCCS-GIST-15 | Gastric | Metastatic | None | KIT Exon 11 c.1668_1691del | IDH2 c.435dup | 0.68 |
| NCCS-GIST-16 | Gastric | Metastatic | None | KIT Exon 11 c.1669_1683del | IDH2 c.435dup | 1.03 |
| NCCS-GIST-17 | Gastric | Metastatic | Imatinib | KIT Exon 11 c.1673_1687del | | |
| NCCS-GIST-18 | Gastric | Metastatic | Imatinib | KIT Exon 11 c.1655_1666del | | |
| NCCS-GIST-19 | Gastric | Metastatic | None | KIT Exon 11 c.1656_1679del | | |
| NCCS-GIST-20 | Small bowel | Metastatic | Imatinib | KIT Exon 11 c.1676T>A | | |
| NCCS-GIST-21 | Small bowel | Metastatic | Imatinib | KIT Exon 11 c.1653_1670del | | |
| NCCS-GIST-22 | Small bowel | Metastatic | Imatinib | KIT Exon 11 c.1669T>C | | |
| | | | | TP53 Exon 8 c.841G>A | | |
| NCCS-GIST-23 | Rectum | Metastatic | Imatinib | KIT (site unknown) | | |
| NCCS-GIST-24 | Rectum | Metastatic | Imatinib | KIT (site unknown) | | |
| NCCS-GIST-25 | Gastric | Metastatic | Imatinib | KIT (site unknown) | KRAS c.194G>A | 1.1 |
| NCCS-GIST-26 | Small bowel | Metastatic | Imatinib | KIT (site unknown) | KRAS c.175G>A | 1.62 |
| | | | | | KRAS c.186_187delins | 2.33 |
| NCCS-GIST-27 | Small bowel | Metastatic | Imatinib | KIT Exon 11 c.1669_1674del | | |
| NCCS-GIST-28 | Small bowel | Metastatic | Imatinib | KIT Exon 11 c.550-558del | KIT Exon 11 c.1650_1667del | 0.65 |
| | | | | | KIT Exon 11 c.1667A>T | 0.57 |
| | | | | | KRAS c.175G>A | 1.16 |
| | | | | | KRAS c.194G>A | 2.9 |
| | | | | | KRAS c.186_187delins | 1.7 |
| NCCS-GIST-29 | Small bowel | Metastatic | Sunitinib | KIT Exon 11 c.551-557del | | |
| NCCS-GIST-30 | Gastric | Metastatic | Imatinib | KIT Exon 11 c.1669_1674del | KIT Exon 11 c.1706T>G | 0.36 |
| NCCS-GIST-31 | Gastric | Metastatic | Imatinib | KIT Exon 11 c.1679T>A | SETD2 c.3948del | 0.52 |
| NCCS-GIST-32 | Gastric | Metastatic | Imatinib | KIT Exon 11 c.1669_1674del | | |
| NCCS-GIST-33 | Gastric | Localized | Imatinib | KIT Exon 11 c.1717_1758dup | | |
| NCCS-GIST-34 | Gastric | Localized | Imatinib | KIT Exon 11 c.1669_1674del | KIT Exon 11 c.1706T>G | 0.36 |
| NCCS-GIST-35 | Gastric | Localized | Imatinib | KIT Exon 11 c.1670_1675del | IDH2 c.435dup | 0.67 |
| NCCS-GIST-36 | Gastric | Localized | Imatinib | KIT Exon 11 c.557-559del | | |

*(Continued)*

**TABLE 2** | Continued

| Patient ID | Primary site | Disease status | Treatment at time of study inclusion | Known tumor mutation | Mutation in ctDNA | Allele frequency |
|---|---|---|---|---|---|---|
| NCCS-GIST-37 | Gastric | Localized | None | KIT Exon 11 c.1669T>C | | |
| NCCS-GIST-38 | Gastric | Localized | None | KIT Exon 11 c.1676T>A | | |
| NCCS-GIST-39 | Gastric | Localized | None | KIT Exon 11 c.1727T>C | | |
| NCCS-GIST-40 | Gastric | Localized | None | KIT Exon 11 c.1679T>A | | |
| NCCS-GIST-41 | Small bowel | Localized | Imatinib | KIT Exon 11 c.1655_1668delins | | |
| NCCS-GIST-42 | Small bowel | Localized | None | KIT Exon 9 c.501-503ins | | |
| NCCS-GIST-43 | Gastric | Localized | None | PDGFRA Exon 18 c.2525A>T | | |
| NCCS-GIST-44 | Gastric | Localized | None | PDGFRA Exon 18 c.2525A>T | | |
| NCCS-GIST-45 | Small bowel | Localized | None | KRAS Exon2 c.35G>T | | |
| NCCS-GIST-46 | Small bowel | Localized | None | KIT/PDGFRA wild-type | | |

*†Evidence of progression.*

the exon 17 c.2467T>G variant was only found in plasma ctDNA. Although the variant AF for both variants decreased from week 0 to week 8, it did not correlate with decrease in tumor size. For NCCS-GIST-08 (**Figure 3F**), the *KIT* variant previously reported in tumor was not detected in ctDNA from both samplings (week 0 and week 20). A *KRAS* c.194G>A variant was detected, albeit at low AF (0.8%), in the week 20 sampling.

## ctDNA Mutation Detection in Whole Blood Samples

In an exploratory analysis, we attempted to detect known variants (previously found in the tumor) from ctDNA extracted from whole blood rather than from plasma. ctDNA was extracted from whole blood from 7 patients with metastatic GIST (**Supplementary Table 2**). NGS libraries were subsequently constructed from these extracted ctDNA. We compared the result from whole blood with those corresponding plasma-derived ctDNA as well as tumor. *KIT* variant was detected in 1 whole blood sample from NCCS-GIST-02. The *KIT* variant, exon 11 c.1727T>C, was the same as that reported for the corresponding plasma-derived ctDNA and tumor. The AF for this *KIT* variant in whole blood-derived ctDNA was 1.61%. This AF value was approximately 1/12 of the AF value found in plasma ctDNA (20.3%).

# DISCUSSION

Our results showed that known tumor mutations, including both *KIT* and *PDGFRA*, were detectable in ctDNA only among patients with metastatic GIST with measurable disease progression. However, even in this group of patients, the detection of these mutations may depend on tumor burden at the time of progression. In some cases (NCCS-GIST-08 and NCCS-GIST-09), *KIT* mutations in ctDNA remain undetectable despite continued disease progression across serial samplings, suggesting that not all GISTs shed sufficient ctDNA for detection of mutations. Interestingly, as demonstrated by case NCCS-GIST-02 at week 0, despite the primary *KIT* exon 11 c.1727T>C being detected at high AF, the secondary *KIT* exon 17 c.2466T>A was not detected, implying that these mutations may not be shed at the same rates. Subsequent sampling upon disease progression simultaneously identified *KIT* exon 17 c.2466T>A and exon 13 c.1961T>C variants. Regardless, any detected ctDNA mutation became undetectable upon disease response to a subsequent line of TKI therapy. These results are generally consistent with previous reports (10–13). Taken together, these findings highlight the value of ctDNA mutation testing in the setting of progressive disease, enabling the detection of secondary acquired mutations, and facilitating treatment response assessment.

Among the GIST patients who progressed on TKI therapy evaluated in our study, we identified a potentially actionable acquired *PIK3CA* exon 9 c.1633G>A variant in NCCS-GIST-17 upon resistance to imatinib. In a previous study on 529 imatinib-naïve GISTs, only eight primary and two metastatic cases

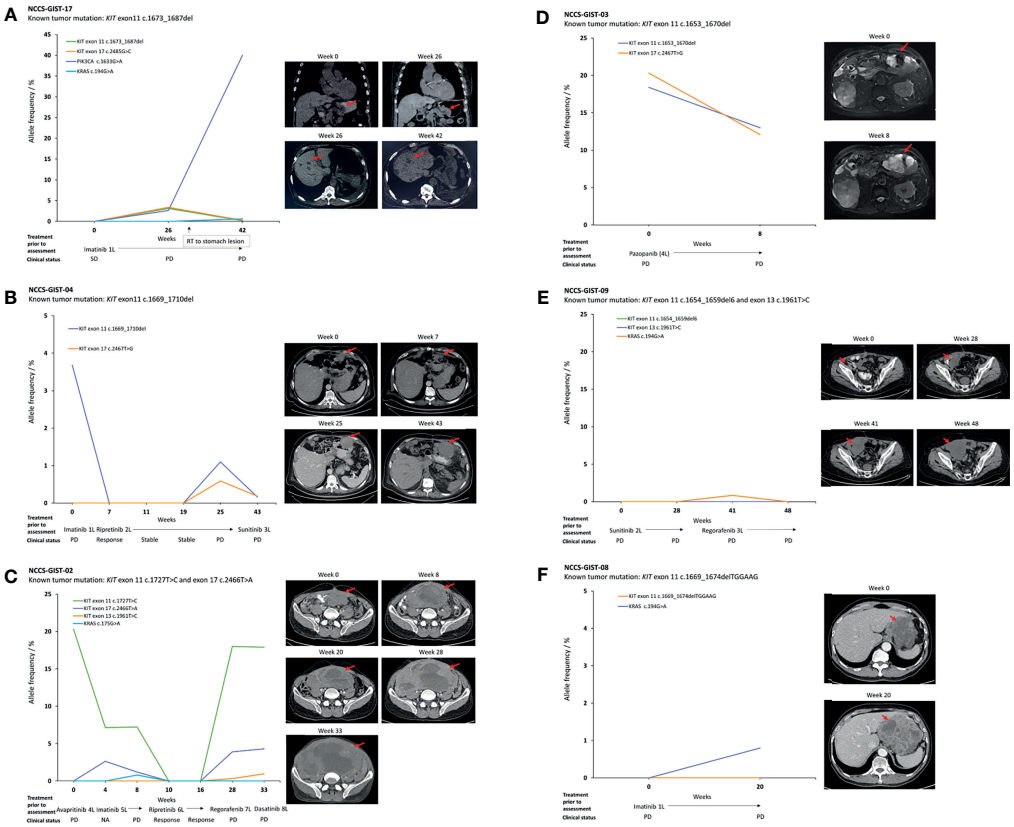


**FIGURE 3** | Temporal correlation of ctDNA mutations and patients with advanced GIST progressing while on TKI therapy. **(A)** Detectable *KIT* exon 11 and 17 mutations, as well as a potentially actionable *PI3K3CA* c.1633G>A mutation upon disease progression. The *PI3K3CA* variant was dominant over other mutations in ctDNA on further disease progression. **(B–D)** Trajectory and correlation of ctDNA variants with disease status. **(E, F)** Non-detection of *KIT* mutations in ctDNA despite disease progression. Each figure is accompanied with the CT scans taken at the indicated time points. The red arrows refer to the location of the tumor. SD, stable disease; PD progressive disease.

harbored *PIK3CA* mutations, though these cases tended to be large (>10 cm) (14). These results suggest that *PIK3CA* mutations may confer growth advantages in GISTs and may form the dominant clone in the setting of imatinib resistance. Consequently, this offers an opportunity for therapeutic intervention using PI3K inhibitors (15). Interestingly, we also observed the occurrence of mutations in the histone modifier gene *SETD2* in three patients. Previously, Huang et al. demonstrated somatic alterations of *SETD2* in 10 out of 89 (11.2%) high-risk/metastatic GIST cases but not low-/intermediate-risk cases. In gastric GISTs, *SETD2* mutations were associated with hypomethylated heterochromatin and worse relapse-free survival (9). The ability to identify predictive and prognostic biomarkers in a non-invasive manner is an attractive feature of liquid biopsy-based testing, though the actual clinical utility will require validation in a larger cohort.

Several liquid biopsy-based assays deployed for detection of *KIT* or *PDGFRA* mutations have previously been reported in GISTs, including allele-specific ligation PCR (10), digital droplet PCR (11), BEAMing (16), and targeted amplicon sequencing (12, 17). Although assays targeting single mutations are highly sensitive, they are not easily generalizable in GISTs as they are characterized by a range of primary and secondary mutations. In our study, we deployed a customized targeted panel (Archer® LiquidPlex™) comprising 29 cancer and/or GIST-associated genes, emphasizing the utility of a larger sequencing footprint in picking up clinically relevant mutations beyond *KIT* and *PDGFRA*. Similar to a previous report (18), the sensitivity of our assay depends on tumor size, and is most applicable in the setting of progressive disease and TKI resistance. An additional limiting factor in the detection of ctDNA in our study may include the extent of vascularization of individual tumors, which would determine the ease of accessibility into the blood system for the ctDNA. We do take note that some of our plasma samples have been in storage at −80°C for more than the latest recommended storage period of 9 months (19). Among the 7 out of 10 patients with progressing metastatic GIST with significant detectable ctDNA mutations, 4 of the plasma samples were stored between 8 and 10 years, while the rest ($n$ = 3) were stored for less than 1 year. In this case, storage period may not be the main issue. It is likely that when stored properly at the right temperature and repeated freeze–thaw is not allowed, plasma can be stored for a longer period. A previous report suggests the feasibility of using small volumes of dried whole blood spots for ctDNA mutation

detection (20), and our exploratory investigation showed that this approach is clearly less sensitive than using plasma ctDNA, and the reagent costs also implies lower cost-effectiveness.

In conclusion, detection of GIST-related mutations in ctDNA using a customized targeted NGS panel represents an attractive non-invasive means to obtain clinically tractable information.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by SingHealth Centralised Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

TK and JC analyzed the data and drafted the manuscript. TK, EL, CN, and BT provided technical advice and experimental support. VY, MF, and JC obtained patient data and contributed samples. JC and NS designed the study, interpreted the results, and revised the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by the ArcherDX Merit Grant Award (ArcherDX LLC is a subsidiary of Invitae Corporation), Singapore Ministry of Health's National Medical Research Council Research Training Fellowship (NMRC/Fellowship/0054/2017), SHF-Foundation Research Grant (SHF/FG653P/2017), Agency for Science, Technology and Research (A*STAR) IAF-PP Grant (H18/01/a0/019), as well as the SingHealth Duke-NUS Academic Medical Centre and Oncology ACP (08-FY2017/P1/14-A28, 08/FY2020/EX/08-A43, and 08/FY2020/EX/75-A151). The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

## ACKNOWLEDGMENTS

The authors would like to thank all patients for their participation in this study.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.840843/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 24 March 2022
doi: 10.3389/fonc.2022.843561

# Genetic Characteristics Associated With Drug Resistance in Lung Cancer and Colorectal Cancer Using Whole Exome Sequencing of Cell-Free DNA

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**Specialty section:**
This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology

**Received:** 26 December 2021
**Accepted:** 01 March 2022
**Published:** 24 March 2022

**Citation:**
Lee JW, Park YS, Choi JY, Chang WJ, Lee S, Sung JS, Kim B, Lee SB, Lee SY, Choi J and Kim YH (2022) Genetic Characteristics Associated With Drug Resistance in Lung Cancer and Colorectal Cancer Using Whole Exome Sequencing of Cell-Free DNA. Front. Oncol. 12:843561. doi: 10.3389/fonc.2022.843561

Circulating cell-free DNA (cfDNA) can be used to characterize tumor genomes through next-generation sequencing (NGS)-based approaches. We aim to identify novel genetic alterations associated with drug resistance in lung cancer and colorectal cancer patients who were treated with EGFR-targeted therapy and cytotoxic chemotherapy through whole exome sequencing (WES) of cfDNA. A cohort of 18 lung cancer patients was treated with EGFR TKI or cytotoxic chemotherapy, and a cohort of 37 colorectal cancer patients was treated with EGFR monoclonal antibody or cytotoxic chemotherapy alone. Serum samples were drawn before and after development of drug resistance, and the genetic mutational profile was analyzed with WES data. For 110 paired cfDNA and matched germline DNA WES samples, mean coverage of 138x (range, 52–208.4x) and 47x (range, 30.5–125.1x) was achieved, respectively. After excluding synonymous variants, mutants identified in more than two patients at the time of acquired resistance were selected. Seven genes in lung cancer and 16 genes in colorectal cancer were found, namely, APC, TP53, KRAS, SMAD4, and EGFR. In addition, the GPR155 I357S mutation in lung cancer and ADAMTS20 S1597P and TTN R7415H mutations in colorectal cancer were frequently detected at the time of acquired resistance, indicating that these mutations have an important function in acquired resistance to chemotherapy. Our data suggest that novel genetic variants associated with drug resistance can be identified using cfDNA WES. Further validation is necessary, but these candidate genes are promising therapeutic targets for overcoming drug resistance in lung cancer and colorectal cancer.

**Keywords: circulating cell-free DNA (cfDNA), whole exome sequencing, drug resistance, lung cancer, colorectal cancer**

## INTRODUCTION

Significant progress has been made for tracking tumor mutations in cell-free DNA (cfDNA) in the last decade (1–3). cfDNA is thought to be released into circulation by necrotic or apoptotic cells, and is frequently present at higher quantities in patients with cancer than in healthy individuals (4). An analysis of those cfDNA can be used as a surrogate marker for molecular diagnosis, and for surveillance of tumor progression (5). These techniques enable an access of tumor molecular information when tumor biopsies are intractable, particularly for patients with metastatic cancer.

Whole exome sequencing (WES) of cfDNA has demonstrated potential to detect clinically relevant alterations (6). Although significant progress has been made for tracking previously detected tumor mutations using targeted gene panels or single gene assays, WES enables a more comprehensive analysis covering the complex landscape of somatic alterations (7). Also, WES analyses of cfDNA hold great promise to identify emerging genetic alterations that are of interest in treatment of drug resistance.

Lung cancer and colorectal cancers are the two leading cancer causing mortalities in both men and women in Korea during 2021 (8). Lung cancer and metastatic colorectal cancer (mCRC) are often diagnosed at an advanced stage when tumor cell dissemination has taken place (9). The treatment of the RAS wild-type mCRC is currently based on the use of chemotherapy doublets (fluoropyrimidine and oxaliplatin or irinotecan) and biological drug (cetuximab, bevacizumab, panitumumab) (10). This concept is well expressed in the ESMO (European School of Medical Oncology) guidelines (11). In lung cancer, the use of EGFR tyrosine kinase inhibitors (EGFR-TKIs) is now a common practice for the first-line treatment of patients with EGFR sensitizing mutation, leading to longer progression-free survival (PFS) intervals with fewer or at least different side effects than chemotherapy (12). Mechanisms of acquired resistance to targeted therapy in both types of cancer have been largely deciphered over the past 20 years and targeting those genetic driver changes are already in clinical use or under clinical investigation (13). Despite great promises brought by the new paradigm of targeted therapy, the invariable emergence of acquired drug resistance not only limits the duration of tumor response but also represents the major obstacle for more meaningful impact on long-term survival in genotype-matched precision medicine (14). The study of sequential liquid biopsies, obtained at baseline and at the moment of progression, from lung cancer and mCRC patients has allowed the identification of new genetic alterations, which explain the development of acquired resistance.

There are a few reports of attempts to analyze cfDNA WES data as a platform for non-invasive analysis of tumor evolution during cancer treatment (4, 5, 15). Yet, the study with a large number of patients in lung cancer and colorectal cancer was never investigated. Our purpose in this investigation was to perform WES of serum cfDNA in patients with lung cancer and colorectal cancer. From the analysis of those data, novel genetic variants associated with drug resistance could be identified.

## MATERIALS AND METHODS

### Patients and Sample Selection

Patients with metastatic colorectal cancer (mCRC) and advanced lung cancer who had been treated with adjuvant chemotherapy at the Korea University Anam Hospital and Guro Hospital were reviewed. From January 2010 to December 2019, patients were treated with chemotherapy (Lung cancer—EGFR TKI or Cytotoxic chemotherapy), (Colorectal cancer—Cytotoxic chemotherapy or Cetuximab) and total of 42 lung cancer patients and 63 colorectal cancer patient samples were used for this study if serum samples for WES of the baseline and at acquired resistance were both available. Both blood samples from baseline and resistance time points were used for cfDNA extraction and WES. Tumor response was determined in accordance with the Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) guidelines (16). Tumor size was measured using summation of the longest diameter of two largest tumors. If a lesion is smaller than 5 mm, it was recorded as non-measurable. The study was approved by the institutional review boards of the Korea University Anam Hospital and Guro Hospital, and informed consent was obtained.

### Blood (Serum) Sampling

Blood samples were obtained at diagnosis and subsequently in several-month intervals during treatment and follow-ups. Blood samples were collected in SST Vacutainer tubes (Yellow top) for serum isolation. For serum isolation, tubes were centrifuged at 2,500 rpm for 7 min at RT, and the supernatant fraction transferred to a fresh tube and re-centrifuged at 16,000×*g* for 10 min at 4°C. The supernatant fraction from the second centrifugation was transferred to a cryotube for storage in a −80°C freezer in our laboratory within 1 to 4 h after collection.

### cfDNA and Germline DNA Extraction

cfDNA was extracted from 1 to 2 ml of serum using a Qiagen circulating nucleic acid kit (Qiagen,Germany) with the Quavac24s system, according to the recommendation of the manufacturers. When required, additional purification was performed using Agencourt AMPure XP (BeckMan Coulter, Brea, CA) to remove larger contaminating nucleic acid. cfDNA concentration and quality were measured by Tapestation or Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) using the High sensitivity DNA kit. Germline DNA was extracted from PBMCs using Qiagen blood minikit (Qiagen) according to the instructions of the manufacturer.

### Library Preparation and WES

For cfDNA library preparation, an average 10 ng of cfDNA were engaged without an initial fragmentation and Agilent SureSelect Human All Exome V4 Kit and Twist Human Core Exome Kit were used according to instructions of the manufacturer. WES was performed on serum samples from 105 patients using an Illumina HiSeq 2500, with 100-bp paired-end reads.

### Bioinformatic Analysis (Pipeline)

Sequence QC was done through FastqQC 0.11.2 (17), and it was mapped to human reference genome sequence NCBI b37 using

bwa 0.7.12 (18). BAM files were realigned with the Genome Analysis Toolkit 4.1 (19) (GATK) IndelRealigner, and base quality scores were recalibrated by the GATK base quality recalibration tool. WES variants calling was performed using two variant callers with 1% cut off value: GATK's Mutect2 v4.1.4.1 (20) return only SNVs and Strelka2 (21) returns the lowest number of both SNV and indel calls according to their somatic pipeline, respectively. Final variants were annotated using ANNOVAR-v2021-06-07 with build hg19 databases, namely, refGene, dbNSFP version 2.6, COSMIC database version 70, NHLBI-ESP project with 6,500 exomes, 1000 Genomes Project, dbSNP 138, CLINVAR database, Polypen2, COSMIC, ICGC and functional prediction was performed. SNVs with quality <30, a depth of coverage <20 in cfDNA samples, or <3 reads supporting the variant were filtered out. Only within exons of coding genes or splicing sites were kept. Then, variants reported in more than 1% of the population in the 1,000 genomes or Panel of Normal of Exome Sequencing of Korean population (22) were discarded to filter out polymorphisms. Finally, synonymous variants were filtered out except those with a COSMIC ID. Subsequently, all identified somatic mutations were manually examined by visual inspection of the BAM files to remove false positive calls as were located in repetitive areas and variants with many adjacent variants as they were suspected to result from systemic misalignment.

Copy number variation analysis was performed using FACETS V0.5.6: https://github.com/mskcc/facets (23). ctDNA fraction was estimated by FACETS from data of the WES cfDNA sample and absolute copy number (ABCN) were called depends on tumor fraction estimation from cfDNA as previously described (24), and mean tcn.em values were used. To estimate ctDNA amount, mean tcn.em values were used to calculate ctDNA content of total cfDNA (25).

Mutational signature analysis was performed using the deconstructSigs package in R (26), Signal (27) or MuSiCa (28) that selects which combination of known mutational signatures can account for the observed mutational profile in each sample as previously described (29).

## ddPCR

Mutant allele frequency was assessed using the QX200 Droplet Digital PCR (ddPCR) System (BioRad, Milan, Italy) in accordance with the instructions of the manufacturer. The PrimePCR™ ddPCR™ Mutation Assay (BioRad) for humans was used. This kit evaluates KRAS p.G12C and KRAS WT for p.G12C, KRAS p.G12R and KRAS WT for p.G12R, KRAS p.G12V and KRAS WT for p.G12V, KRAS p.G12D and KRAS WT for p.G12D, KRAS p.G12S and KRAS WT for p.G12S, KRAS p.G13D and KRAS WT for p.G13D, KRAS p.G13C and KRAS WT for p.G13C, NRAS p.Q61R and NRAS WT for p.Q61R, EGFR p.E746_A750del and EGFR WT for p.E746_A750del, EGFR p.L858R and EGFR WT for p.L858R, and BRAF p.V600E and BRAF WT for p.V600E. ddPCR reaction mixtures contained a final concentration of 250 nM of each of the probes, 900 nM of forward and reverse primers, 1× ddPCR Supermix for Probes (Bio-Rad), and 0.7–3 ng cfDNA in a final volume of 20 µl. Each reaction included a blank sample corresponding to H2O, another corresponding to wild-type DNA, and a positive control (KRAS p.G12D, EGFR p.E746_A750del and EGFR p.L858R) using HD780 Reference Standard Set (Horizon, Cambridge, UK). The steps are described in more detail as previously (30).

## Statistical Analysis

Statistical analysis was performed using R software (version 4.0.3). Pearson's correlation coefficient R >0.5 was considered to indicate a strong correlation. Survival curved were plotted using the cBioPortal and Kaplan–Meier plots as previously described (31). All results are displayed with P-values from a log-rank test. A P-values of <0.05 were considered to be statistically significant.

# RESULTS

## Overall Study Design and Patient Characteristics

The study comprised a major aim to identify the somatic variants associated with drug resistance to chemotherapy in lung cancer and colorectal cancer using circulating cell free DNA. For this purpose, we performed the whole exome sequencing of serum samples from 55 patients with stage III or IV cancer (18 lung cancer and 37 colorectal cancer patients) which were drawn before and after the development of drug resistance. Patient characteristics, namely, clinical and histological features in this study are detailed in **Table 1**. The mean age was 65 and 64 years old in lung cancer and colorectal cancer, respectively. In lung cancer, all tumor types being treated were non-small cell lung cancer and the patients received standard cytotoxic chemotherapy (38.9%) or EGFR-TKI (61.1%). In colorectal cancer, all the tumor types being treated were adenocarcinoma and all patients were treated with a modified standard cytotoxic chemotherapy (78.4%) or additional EGFR monoclonal antibody cetuximab (21.6%). With this approach, we could expect to find not only drug specific genetic variants, but also common variants regardless of drug type.

## cfDNA WES Analysis and Bioinformatics Pipeline

A total of 210 cfDNA paired samples underwent WES, and it generated for a median 83.5x coverage (range, 20.1–211.5x). For samples over 70x coverages, there were few PCR duplicates, so re-sequencing was performed on those samples that can be expected to increase mean coverage up to 100x. A total of 152 samples (59 in lung cancer + 93 in colorectal cancer) had a median of 91.5x and the rest had a median of 51.8x. Thus, we re-sequenced 152 samples to achieve a median 130.5x (mean = 134.5x) and was used for the downstream analysis. Pair analysis was performed with samples with WES at mean coverage of 80x or more at both baseline and at the time of acquired resistance and gap between the two value less than 30x. Finally, this study included 110 paired serum samples from 18 lung cancer and 37 colorectal cancer patients which were drawn before and after the development of drug resistance. For those 110 paired cfDNA and

**TABLE 1** | Patient characteristics.

| | Lung cancer (n = 18) | | Colorectal cancer (n = 37) | |
|---|---|---|---|---|
| | Cytotoxic chemotherapy N = 7 | EGFR-TKI N = 11 | Cytotoxic chemotherapy only N = 29 | Chemotherapy with Cetuximab N = 8 |
| **Sex** | | | | |
| Female | 3 | 4 | 12 | 2 |
| Male | 4 | 7 | 17 | 6 |
| **Age at diagnosis (mean years ±SD)** | 62 ± 12 | 67 ± 7 | 65 ± 14 | 60 ± 9 |
| **Histology (Lung cancer)** | | | | |
| NSCLC | 5 | 11 | | |
| Adenocarcinoma | 1 | | | |
| Squamous | 1 | | | |
| SCLC | | | | |
| **Histology (Colorectal cancer)** | | | | |
| Adenocarcinoma | | | 29 | 8 |

matched germline DNA WES, a mean coverage of 138x (range, 52–208.4x) and 47x (range, 30.5–125.1x) were achieved respectively, and thus enabling a detection of MAF at 1%. Among these patients, the median age was 65 (range, 24–88) years and there were 34 men (61.8%). Patient enrollment and study overview are presented in **Figure 1** and **Table 1**.

For somatic mutation calling from cfDNA WES data, we used two variants callers GATK's Mutect2 and Strelka2 as described in the *Materials and Methods* section. Circulating Tumor DNA (ctDNA) fraction was estimated by FACETS from the cfDNA WES data and mean cf.em values were used. Analysis based on the FACETS tool revealed a mean 28.2% of ctDNA (range, 17–69.3%) in lung cancer and mean 30.9% of ctDNA (range, 15.7–71%) in colorectal (**Figure 2**). These numbers are comparable to those observed from the cfDNA WES analysis of the other 44 cancer patients (mean 18%, range, 4.5–36.2%) although tumor types are different as metastatic breast and prostate cancer (3). We assessed whether ctDNA content was associated with the number of called somatic mutations or the residual tumor information of the patient. Indeed, we found that the number of somatic variants were associated with ctDNA amount and residual tumor size in colorectal cancer (Pearson's correlation, rho = 0.25, p-value = 0.033 and rho = 0.27, p-value = 0.02, respectively), but not in lung cancer (**Supplementary Figure S1A**). The total amount of ctDNA did not show significant differences depending on the residual tumor size (**Supplementary Figure S1B**). This result indicates that correlate ctDNA with a number of somatic variants was well reflected in colorectal cancer than lung cancer, and there are similar findings observed in a study of ctDNA with various tumor types (32, 33).

For the 36 serum samples from 18 lung cancer patients, we identified a mean 43.7 SNVs and small indels (range, 5–487). Also, for the 74 serum samples from 37 colorectal cancer patients, a mean 60.5 SNVs and small indels (range, 3–243) were identified. As a result, we identified a total of 1,576 somatic variants in 36 lung cancer serum samples (**Figure 2A**), and 4,480 somatic variants in 74 colorectal cancer serum samples (**Figure 2B**). These numbers are comparable to those observed in one of the largest studies attempted at WES-based TMB quantification from liquid biopsy (mean 140 variants, range,

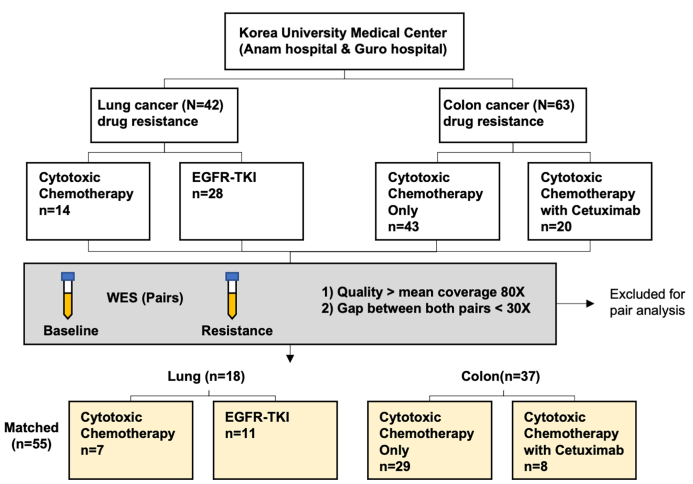


**FIGURE 1** | Patient recruitment and enrollment flow chart.

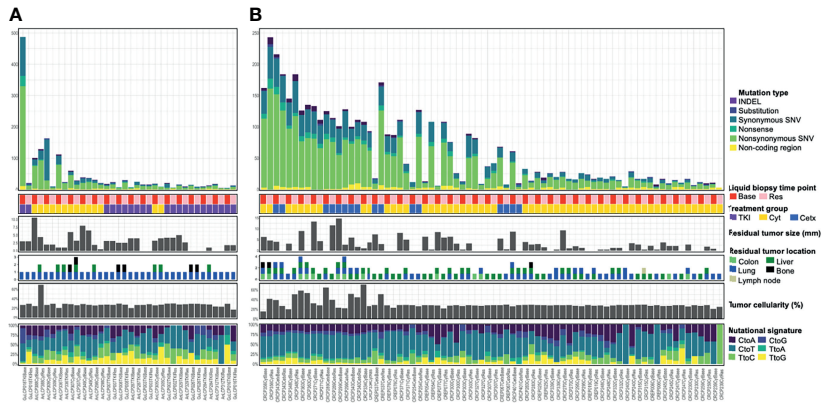


**FIGURE 2** | Somatic mutational landscape across lung cancer **(A)** and colorectal cancer **(B)** cfDNA samples analyzed with WES. Top bar graph showed a distribution of somatic mutation count across cfDNA samples in 18 lung cancer patients **(A)** or in 37 colorectal cancer patients **(B)**. The second matrices show liquid biopsy time point or treatment group. Base, baseline; Res, Resistant time point; TKI, EGFR-TKI; Cyt, Cytotoxic chemotherapy; Cetx, Cetuximab. The third bar graphs illustrate residual tumor size, location, and tumor cellularity as a proportion of total cfDNA. The bottom bar graph shows transcriptomic data composition. Samples are ordered by patient and mutation count as determined from WES. Some tiny nodules smaller than 5 mm were non-measurable and not be shown in Residual tumor size section.

19–818) which was performed on 32 metastatic patients with various cancer types (34) considering that the amount of input DNA used is half. The samples of the each cohort exhibited outlier highest number of somatic mutation (243–487 SNVs and small indels; 4.8–9.5 Mut/Mb) and can be considered to exhibit a 'Hypermutator-like condition' as described in the TCGA study including lung cancer (35). Among an updated inventory of about 276 human DNA repair genes (36), two samples (GuLCP018TKIBase and CRCP343CetxBase) showed more than twelve DNA repair gene variants (**Supplementary Table 1**). Moreover, 23 DNA repair gene variants including BRCA1 were shown in the GuLCP018TKIBase sample.

Next, we examined the composition of six possible base-pair substitutions and found that a high rate of C > T transition, for all groups (**Supplementary Figure S2A**). Consistently on decomposing the mutational spectrum is similar to the trinucleotide signature associated with aging (e.g., COSMIC signature 1) and defective DNA MMR (e.g., COSMIC signature 15), a mutational process that is prevalent in most lung cancer and colorectal cancers (37). The median proportion of signatures 2 and 13 (APOBEC) was higher and signature 24 (Aflatoxin) was lower in baseline group compared with the resistance to EGFR-TKI group in lung cancer.

In a recent study using endometrial cancer, the researchers detected acquired high MSI in ctDNA from one patient whose primary tumor was MSI stable (38). We analyzed the MSI and found that no samples had more than 3% unstable microsatellites (**Supplementary Figure S2B**). These results indicate that those two samples with high number of somatic mutations was potentially explained by DNA repair gene alteration.

## Validation of cfDNA WES Using ddPCR

To validate the dynamic range and accuracy of WES, a subset of samples with cfDNA availability was tested by ddPCR for mutation detection in 4 genes (KRAS, NRAS, EGFR, and BRAF). A total of 14 samples were applied to select variants having at least one read containing the allele of the variants. Orthogonal validation with serum ddPCR for those mutations showed concordant findings to serum WES in 10 of 14 results, and the ddPCR-derived VAFs correlated well with those obtained with WES (Pearson's correlation = 0.97, P-value = 2.7e−08; **Supplementary Figure S3**). Also, it indicated a high accuracy (12/14 = 85.7%) of ddPCR measurement for those probes with a level of mutant fractional abundance ≥1%. Thus, we applied bioinformatics pipeline that enabled to establish a threshold for SNV detection of 1% by cfDNA WES, below which SNVs were not distinguishable from the background.

## Identification of SNVs Associated With Drug Resistance in cfDNA

To evaluated whether the somatic variants in cfDNA related with drug resistance could be identified, the variants at the time of acquired resistance were compared to those in baseline. To identify somatic variants, germline DNA from PBMCs was used as a control. Germline variants and acquired somatic alterations from clonal hematopoiesis are estimated to be removed during this process. After checking the bam files and plot reads and removing false positive, we found a median of 18.5 mutations in lung cancer and 26 mutations in colorectal cancer per patients. After excluding synonymous variants, we selected genes that were changed during observation at the time of baseline only or acquired resistance only. Likewise, increased or decreased VAF over 5% genes with more than two cancer patients were selected. This yielded seven genes in lung cancer, and 16 genes in colorectal cancer, which are plotted in a heat map with one-way hierarchical clustering referring to treatment conditions as shown in **Figure 3**.

Several mechanisms of resistance have been described to anti-EGFR-TKI in lung cancer and anti-EGFR monoclonal antibody in colorectal cancer (39–42). Of those, KRAS, EGFR, and APC

were analyzed and KRAS (G12D, G12V), EGFR (Ex19 del, L858R) and APC R213X were detected in seven patients. EGFR Ex19 del and L858R mutations were detected at baseline time point only in three lung cancer patients. Samples with those three patients showed similar tumor cellularity both at baseline and acquired resistance time point and VAF might be not affected by tumor cellularity. This result indicates that clones with those EGFR mutations could be decreased and other resistant clones were expanded.

KRAS G12C, C12V, and G12D variants were detected at resistance time point only or increased in three colorectal cancer patients who were treated with cytotoxic chemotherapy. Various APC nonsense mutations were identified in both baseline and resistance time points. Among them, Hotspot mutation R213X was increased or observed at resistance time point only. These results indicate that previously reported variants related to drug resistance could be identified in cfDNA WES.

## Investigation of Top Frequently Mutated Genes

To evaluate the somatic variants potential for drug resistance, we first focused on the frequently mutated genes in cfDNA with acquired resistant time point. In lung cancer, TP53 gene harbored three mutations, NACA2 and GPR155 genes had two mutations, and VNN1 gene possessed one mutation (**Supplementary Table 2**). Genetic alteration of these genes was visualized as an oncoprint representing missense mutations, nonsense mutations, and non-frameshift substitution (**Figure 3**). Lung cancer patient data and the cBioPortal online tool were used for examine these mutated genes. Among them, GPR155 I357S mutations were estimated as pathogenic (score 0.99) in the COSMIC database, which were not reported in ClinVar. Interestingly, patients with GPR155 alteration showed short overall survival compared those with unaltered patients (**Figure 4A**). In addition, we found that three of four mutations of GPR155 were located in the I357S position; whereas, other genes contained mutations at multiple locations. Detailed mutation sites in GPR155 are shown in **Figure 4B**. Also, GPR155 mutations were observed only in the EGFR-TKI treated group. This result suggests the possibility that GPR155 I357S mutation may contribute to the drug resistance in lung cancer patients especially EGFR-TKI.

In colorectal cancer, TP53, TTN, OBSCN, and MUC17 genes harbored four mutations: MUC16 had five mutations, and HLA-DRB1, ADAMTS20, and HDAC6 genes possessed three mutations. Remaining NRXN3 and BAGE2 genes had two and one mutations respectively (**Supplementary Table 2**). Genetic alteration of these genes was visualized as an oncoprint representing missense mutations, nonsense mutations, non-frameshift substitution, and untranslated region (**Figure 3**). Except well-known cancer related genes TP53, KRAS, and APC, pathogenic variants were estimated in ADAMTS20 S1597P and TTN R7415H only (scores 0.83 and 0.74 respectively). ADAMTS20 was found to be downregulated in colorectal cancer (43) and TTN was reported to be frequently detected in solid tumors including colorectal cancer (44, 45). This result suggests that those gene alterations may lead to the resistance to chemotherapy in colorectal cancer patients.

## Copy Number Variants (CNVs)

To evaluates whether the somatic CNVs in cfDNA related with drug resistance could be identified, the variants at the time of acquired resistance were compared to those in baseline. Significantly amplified peaks for those two groups were identified using FACETS as described in the *Materials and Methods* section. To select samples with top amplified regions, mean tcn.em values larger than 8 are categorized as "gain". Here, we identified CNVs in cfDNA of lung cancer and colon cancer patients with WES and found that gains in chromosomes 1, 6, 7, 8, 10, 14, 16, 19, and 20. Among them, regions including cancer related genes annotated by Oncomine and Cosmic567 were

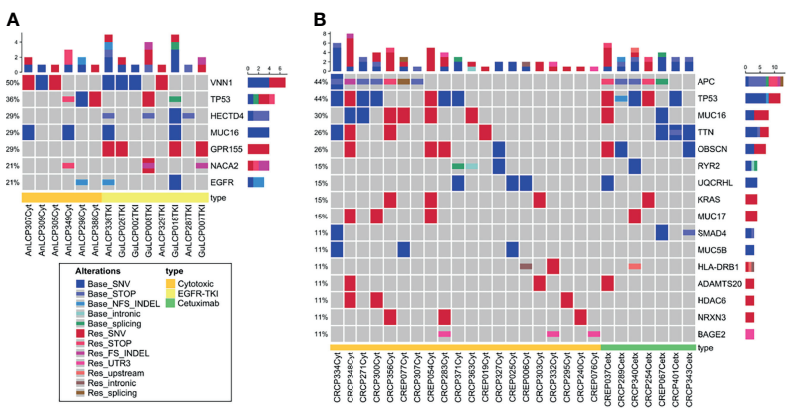


**FIGURE 3** | Oncoprint across lung cancer **(A)** and colorectal cancer **(B)** cfDNA samples analyzed with WES. **(A)** Seven frequently mutated genes in 18 lung cancer patients identified through cfDNA WES. **(B)** Sixteen frequently mutated genes in 37 colorectal cancer patients identified through cfDNA WES. This visualization provides an overview of the non-synonymous alterations in particular genes (rows) affecting particular individual patients (columns). Reddish colors indicate increased VAF over 5% or observed at the time point of acquired resistance only (Res_). Bluish colors decreased VAF over 5% or observed at the time of baseline only (Base_).

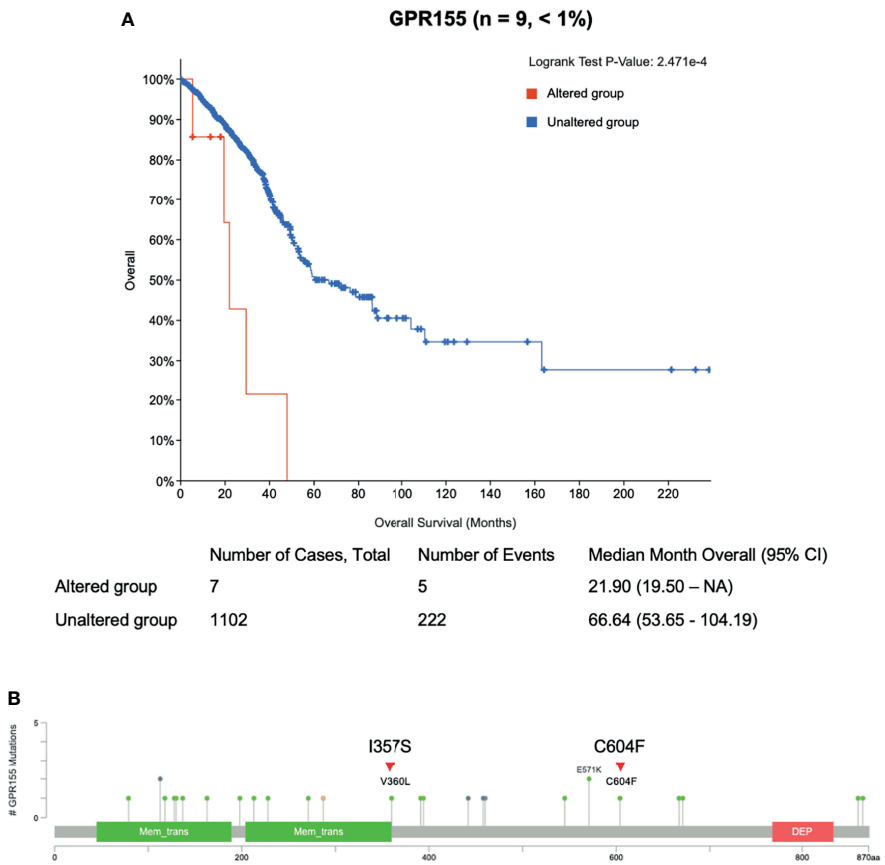


**FIGURE 4** | Clinical relevance of GPR155 mutation in lung cancer patients. **(A)** Overall survival analysis of patients with GRP155 alterations (red line) is compared to that of those without alterations (blue line). **(B)** Different mutation sites of GPR155 in lung cancer. Known hotspot mutation sites in COSMIC data are labeled. Each lollipop label shows the amino acid change and its location in the amino acid sequence. Known gene/protein domains are shown in color, and other regions are colored dark gray. Red triangles represent mutations found in this study, I357S (n = 3) and C604F (n = 1).

selected (**Figure 5** and **Supplementary Table 3**). In the case of AnLCP388Cyt, we observed focal amplification of the 8q24 and 8p11 chromosomal region in an acquired resistant time point. This region containing MYC and FGFR1 genes, and MYC copy number gains in the patients with primary resistance were reported as higher than in the sensitive patients against EGFR-TKI treatment (46). FGFR1 was frequently amplified in squamous cell lung cancer and this indicates that the mechanism of acquired resistance in this patient might be the activation of pathway through MYC and FGFR1 (47). In case of AnLCP336TKI, focal amplification of the 14q13 chromosomal region was observed only in baseline time point. This region containing NKX2-1 and NKX2-8 genes were reported as prognostic factors in lung cancer (48). In the case of CRPC363Cyt, focal amplification of the 19q12 chromosomal region including CCNE1 was also observed in baseline only. In the case of CRCP299Cetx, focal amplification of the 6p21 chromosomal region including CCND3 was observed in both baseline and acquired resistance time points, which suggests that different resistance mechanism would be involved.

**Review Table**

Novel genetic variants associated with drug resistance could be identified through cell-free DNA (cfDNA) whole exome sequencing in lung cancer and colorectal cancer patients.
For cfDNA WES, mean coverage of 138x (range, 52–208.4x) was achieved, and a threshold for SNV detection of 1% was established by ddPCR validation.
GPR155 I357S mutation in lung cancer and ADAMTS20 S1597P and TTN R7415H mutations in colorectal cancer were frequently detected at the time of acquired resistance.
Increased detection indicates that these mutations may have an important function in acquired resistance to chemotherapy.

## DISCUSSION

In this work, we used WES based on cfDNA liquid biopsy for 55 lung and colorectal cancer patients to identify novel somatic variants associated with drug resistance after treatment including cytotoxic chemotherapy or EGFR targeted therapy. Recently, a wide range of genomic alterations have reported association with

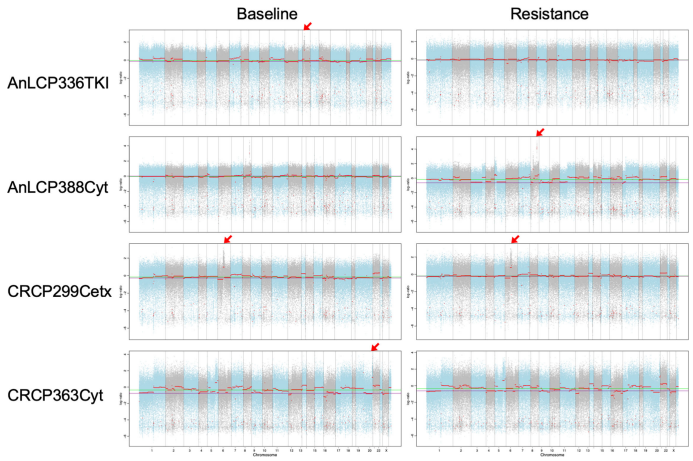


**FIGURE 5** | Copy number variants detected in cfDNA using WES from AnLCP336, AnLCP338, CRCP299, and CRCP363. The red arrows indicate the top amplified region including cancer-related genes annotated by Oncomine and Cosmic567.

cancer behavior, and most of the alterations are generally found in coding regions (49). Therefore, WES is a rational strategy for identifying novel somatic variants associated with drug resistance. To identify somatic variants only, alterations that could possibly be regarded to come from a normal cell were excluded during our analysis using matched normal sample and previous SNP databases. Variants resulting from clonal hematopoiesis could be estimated to be removed using the sequencing results from matched leukocytes as a reference.

It is hypothesized that cfDNA is released from tumor cells through various cell physiological events such as apoptosis, necrosis and secretion into the blood circulation (50). Numerous studies have shown that tumor-derived cfDNA better reflects the complete genetic landscape of the tumor compared to tissue biopsies. Apart from also offering the additional benefits of longitudinal sampling, the analysis of cfDNA represents a promising modality for sequential monitoring of the molecular response of cancer during targeted therapy (51). However, cfDNA profiling also has limitations. Although it is possible that some patients did not have alterations in gene covered by the NGS assay, in most cases, the lack of detection of genomic alterations in cfDNA was likely due to other factors, namely, low tumor burden, lack of cfDNA shedding by some tumors, and timing of blood collection (52). The major technical issue with this approach has been assay specificity and sensitivity. A major drawback of cfDNA assay is the low frequency of some of the mutations that occur in tumors. Low sequencing coverage used for WES resulting in false-negative results for cfDNA variants present below the limit of detection (7). To overcome this limitation, Adalsteinsson et al. pre-selected samples with 10% tumor fraction as a cutoff value using ultralow-pass whole-genome sequencing and showed that only 34% of cfDNA samples from metastatic breast- and prostate cancer patients were feasible for WES analysis (3). Technical feasibility of WES of cfDNA in previous studies has been performed on 303 samples, with a median coverage of 137x (range: 43–500x) (7). In our study, we performed WES to obtain mean 100x coverage, but sequencing coverage was highly variable, ranging from 20.1 to 211.5x coverage, in total 210 paired cfDNA samples. To achieve mean 100x coverage, re-sequencing was performed on sample that could expect increased coverage with low PCR duplicates. Finally, 152 samples were used to achieve a median 130.5x (mean = 134.5x) and 110 paired serum samples were used for the downstream analysis.

Several studies have compared between serum and plasma as use of ctDNA sources (53, 54). The cfDNA yield was higher in plasma from patients with lung cancer or colorectal carcinoma than in healthy controls. Although mutations were identified in both plasma and serum and the median molecular sequencing depth was comparable, more mutations were found in plasma than in serum and the allele frequency was higher in plasma than in serum. Those reports suggest that plasma is clearly more preferable for prospective clinical applications of liquid biopsy. But when our study had started, serum was chosen because it showed higher amount total cfDNA than plasma. Thus, the result of somatic variants calling and allelic fraction might be affected due to being diluted by DNA of non-cancerous origins.

The amount of cfDNA released by tumors is not only dependent on size, but also on turnover activity, proliferation rate, vascularization, and perfusion (51). Therefore, different tumor types of the same size can release different amounts of cfDNA. Bettegowda et al. reported that a fraction of patients with detectable ctDNA varied with tumor type (32). In this study, serum cfDNA were drawn before and after the development of drug resistance of lung and colorectal cancer patients. Although there was no significant difference between lung and colorectal cancer, mean cfDNA amount was higher in the group with larger size of residual tumor (**Supplementary Figure S1B**). It might be affected by the cfDNA from non-cancerous origin as described above. Nonetheless, the number of somatic variants was

associated with ctDNA fraction and residual tumor size in colorectal cancer, but not in lung cancer (**Supplementary Figure S1A**). This could be explained by factors of ctDNA release from the tumor, so-called "ctDNA shed" (55). ctDNA shedding is related not only to tumor size and necrosis, but also the vascularity of tumor. Comprehensive histopathological features of shedding tumors in lung and colorectal cancers were not evaluated. Nevertheless, ctDNA might be released less in lung cancer than in colorectal cancer, due to the physiological conditions such as alveolar region, which is only an efficient region for gas exchange while colorectal tissue has a good network of blood vessels. Besides, multiple metastatic sites that have risen in colorectal cancers may affect more detectable somatic variants than lung cancer. Previously, our group reported that high cfDNA concentrations had significantly shorter PFS and OS than those with low cfDNA concentrations (31). In this study, patients with low ctDNA amount at resistant time point showed longer survival probability but lack statistical significance (**Supplementary Figure S1C**).

Recently, it has been reported that the transformation of EGFR-mutant lung cancer from adenocarcinoma to small-cell lung cancer at the time of acquired resistance is associated with the appearance of APOBEC mutational signatures (56). Isozaki et al. observed increased APOBEC mutational signatures in resistant tumors after TKI treatment and suggest stepwise development of mutations (56). However, no increase in APOBEC mutational signatures was also observed in metastatic sites from a patient with a shorter response to EGFR TKIs (56). In our results, lung cancer patients showed higher APOBEC signature in baseline compared with resistance to the EGFR-TKI group. These results indicate that resistant subclones of our lung cancer patients with EGFR-TKI treated group might be from independent APOBEC-driven clonal evolution during acquired resistance.

It was not surprising to see APC, TP53, KRAS, and SMAD4 as frequently mutated genes in colorectal cancer where such mutations were reported as key driver genes in progression and metastasis (57). Also, TP53 and EGFR have been identified as one of several driver mutations in NSCLC (58), and were frequently detected in our lung cancer samples, indicating the reliability of our current WES study using cfDNA. GPR155 mutation was frequently detected in acquired resistance time point in lung cancer patients only in the EGFR-TKI treated group. GPR155 encodes G protein-coupled receptor 155, and reported that mutations in this gene may be associated with autism (59, 60). Although there has been a report that GPR155 expression is suppressed in neoplasm of the thyroid, hepatocellular carcinoma, and gastric cancer, implying a tumor suppressive function for this gene, the resistant role in lung cancer, however, was not reported (61, 62). In the COSMIC, we found that GPR155 I357S mutations were estimated as pathogenic (score 0.99) and patients with this gene alteration showed poor prognosis compared those with unaltered patients. Hence, it is worthwhile to further investigate the mechanistic roles of GPR155 I357S mutation in drug resistance of lung cancer patients especially EGFR-TKI.

ADAMTS20 gene is a member of the ADAMTS family of zinc-dependent proteases. As an anti-angiogenic member of the family, ADAMTS20 was found to be downregulated in colorectal cancer (43). Mutations in the gene encoding the giant skeletal muscle protein titin (TTN) were reported that associated with several muscle disorders and were frequently detected in solid tumors (44, 63). In colorectal cancer, TTN was identified as the most frequently mutated gene within the pan-cancer cohort, and its mutation number showed the best correlation with TMB (45). Other researchers also observed that TTN, OBSCN, and ADAMTS12 genes were frequently mutated in cfDNA WES although tumor types are different as HCC (64). The association between those mutated genes and drug resistance is not clear in colorectal cancer yet. Recent reports identified that TTN mutations were associated with the largest number of resistant and sensitive drugs (65). Further study of these mutations in colorectal cancer with drug resistance could shed important light on the value of these mutations.

Several genes with high-frequency and important CNVs, namely, MYC, FGFR1, CCNE1, and CCND3 have been observed in lung and colorectal cancer samples. CCNE1 is involved in the cell cycle pathway, and its amplification has been identified in multiple cancers. Among the known driver CNVs found in lung cancer sample, the copy number of MYC and FGFR1 increased in the resistant time point. Schaub et al. described that MYC is the most frequently amplified gene among the proximal network members across all cancer types, and suggest that MYC is a distinct oncogenic driver (66). Increased FGFR1 expression is frequent across various lung cancer histologies, namely, squamous cell carcinomas and adenocarcinomas (67). These genes with CNVs in lung cancer might be potential therapeutic targets.

In conclusion, our study identified the somatic variant associated with drug resistance from lung and colorectal cancer patients using WES and provided a genetic profile. We conclude that cfDNA could be used to identify somatic variants associated with acquired resistance to treatment of lung cancer and colorectal cancer, which could guide change regimen when those biomarkers were detected in the blood.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Korea University Medical Center (ED14110). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Study conception and design: YHK. Patient recruitment and clinical data collection: JYC, WJC, SL, SYL, and YHK. cfDNA isolation and experimental data acquisition: JWL, YSP, JSS, BK, and SBL. WES data analysis and interpretation: JC. Manuscript writing: JWL. Manuscript revision and intellectual contribution: JC and YHK. Study supervision: YHK. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## FUNDING

This research was supported by the Korea Health Technology R&D Project through the Bio & Medical Technology Development Program and Basic Science Research Program of the National Research Foundation funded by the Korean Government, Ministry of Science, ICT and Future Planning (NRF-2015M3A9D7031070, NRF-2017R1A-2B3004624).

## ACKNOWLEDGMENTS

The authors thank the patients and their families. Also, we would like to thank Ms. Sujin Lee for assistance in coordinating consent from patients and in the collection of samples.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.843561/full#supplementary-material

**Supplementary Figure 1 |** Clinical relations of somatic alterations detected in cfDNA from lung and colorectal cancer patients. **(A)** Correlations between ctDNA fraction and clinical information regarding lung cancer and colorectal cancer samples. **(B)** Total Serum cfDNA depending on tumor type and size. Sum of the longest diameter (mm) residual tumors in each type of patient were compared (small = sum of the longest two tumors < 3 cm and large = sum of the longest two tumors ≥ 3 cm). **(C)** Kaplan-Meier estimates of overall survival (OS) according to the amount of ctDNA. Samples were categorized into two subgroups by median ctDNA amount (Lung = 6.65 ng and Colon = 8.88 ng).

**Supplementary Figure 2 |** Characteristics of potential somatic SNVs. **(A)** Comparison between the observed distribution of somatic SNVs across the 96 possible mutation types and summation of the distribution of the decomposed signature. **(B)** Distribution of microsatellite instability (MSI) percentages generated from the cfDNA WES data using msisensor2.

**Supplementary Figure 3 |** Comparing mutation allelic fraction quantified from serum by WES and ddPCR. **(A)** Mutational profile and variant allelic fraction determined by WES and ddPCR in 14 selected serum samples. **(B)** Pearson correlation based on the mutant allelic fraction of the standard in cfDNA samples.

**Supplementary Table 1 |** Somatic variants of 276 DDR genes specific to DNA damage repair pathways in hypermutated samples are listed in 36. Cell Reports.

**Supplementary Table 2 |** Clinical relevance of genes with alterations. Potentially functional and known disease-related variants in the ClinVar and COSMIC databases.

**Supplementary Table 3 |** Genes with CNVs detected in cfDNA.

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frontiers | Frontiers in Oncology

ORIGINAL RESEARCH
published: 12 April 2022
doi: 10.3389/fonc.2022.820510

# Anchored Multiplex PCR Custom Melanoma Next Generation Sequencing Panel for Analysis of Circulating Tumor DNA

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**Specialty section:**
*This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology*

**Received:** *23 November 2021*
**Accepted:** *18 March 2022*
**Published:** *12 April 2022*

**Citation:**
*Diefenbach RJ, Lee JH, Stewart A, Menzies AM, Carlino MS, Saw RPM, Stretch JR, Long GV, Scolyer RA and Rizos H (2022) Anchored Multiplex PCR Custom Melanoma Next Generation Sequencing Panel for Analysis of Circulating Tumor DNA. Front. Oncol. 12:820510. doi: 10.3389/fonc.2022.820510*

Detection of melanoma mutations using circulating tumor DNA (ctDNA) is a potential alternative to using genomic DNA from invasive tissue biopsies. To date, mutations in the GC-rich *TERT* promoter region, which is commonly mutated in melanoma, have been technically difficult to detect in ctDNA using next-generation sequencing (NGS) panels. In this study, we developed a custom melanoma NGS panel for detection of ctDNA, which encompasses the top 15 gene mutations in melanoma including the *TERT* promoter. We analyzed 21 stage III and IV melanoma patient samples who were treatment-naïve or on therapy. The overall detection rate of the custom panel, based on *BRAF/NRAS/TERT* promoter mutations, was 14/21 (67%) patient samples which included a *TERT* C250T mutation in one *BRAF* and *NRAS* mutation negative sample. A *BRAF* or *NRAS* mutation was detected in the ctDNA of 13/21 (62%) patients while *TERT* promoter mutations were detected in 10/21 (48%) patients. Co-occurrence of *TERT* promoter mutations with *BRAF* or *NRAS* mutations was found in 9/10 (90%) patients. The custom ctDNA panel showed a concordance of 16/21 (76%) with tissue based-detection and included 12 *BRAF/NRAS* mutation positive and 4 *BRAF/NRAS* mutation negative patients. The ctDNA mutation detection rate for stage IV was 12/16 (75%) and for stage III was 1/5 (20%). Based on *BRAF*, *NRAS* and *TERT* promoter mutations, the custom melanoma panel displayed a limit of detection of ~0.2% mutant allele frequency and showed significant correlation with droplet digital PCR. For one patient, a novel MAP2K1 H119Y mutation was detected in an *NRAS/BRAF/TERT* promoter mutation negative background. To increase the detection rate to >90% for stage IV melanoma patients, we plan to expand our custom panel to 50 genes. This study represents one of the first to successfully detect *TERT* promoter mutations in ctDNA from cutaneous melanoma patients using a targeted NGS panel.

**Keywords: anchored multiplex PCR, melanoma, circulating tumor DNA, targeted sequencing, custom panel, TERT promoter**

## INTRODUCTION

The analysis of circulating tumor DNA (ctDNA) is progressively being integrated into routine clinical care to monitor cancer recurrence, response to therapy, emergence of resistance and to guide therapy (1–3). In melanoma, ctDNA assessment [reviewed in (4, 5)] predicts overall survival of stage IV melanoma patients treated with BRAF and MEK inhibitors or immunotherapy (6–14) and the relapse-free and melanoma-specific survival of patients with high-risk stage III resected melanoma (15–18). ctDNA can also detect the appearance of treatment-resistant melanoma subclones (6), tumor heterogeneity (19), and metabolic tumor burden (20). ctDNA analysis can inform when to cease therapy (21), predicts disease progression after cessation of immunotherapy (22) and differentiates "true progression" from "pseudoprogression" in melanoma patients treated with immunotherapy (23).

To date, a limited number of studies have employed targeted melanoma next generation sequencing (NGS) panels to analyze mutations in ctDNA (24–29). Targeted ctDNA sequencing panels yielded results concordant with other tissue and liquid biopsy approaches (24–29), and detected ctDNA mutations in 52-74% of stage IV melanoma (24, 25, 27) with 0.1-1.0% limit of detection (LOD). A limitation of these ctDNA panels has been the complete inability to detect *TERT* promoter mutations. This is a significant disadvantage because *TERT* promoter mutations are the most frequent recurrent mutations in melanoma, occurring in 34-80% of cutaneous melanomas and are associated with poor survival (30). The most frequent *TERT* mutations C228T (-124 C>T), C250T (-146 C>T) and CC242TT (138/-139CC>TT) (31–42) occur within high GC DNA regions that is difficult to sequence. As a result, most studies continue to rely on droplet digital PCR (ddPCR), which does not have the multiplexing capabilities of targeted NGS panels, to detect *TERT* promoter mutations (24, 25).

In cutaneous melanoma *TERT* promoter mutations commonly co-occur (80-90%) with *NF1*, *BRAF* or *NRAS* mutations, and TERT promoter mutations are also found in 15-60% of *BRAF/NRAS/NF1* WT cutaneous melanoma background (33, 38–40, 43–46). This highlights the need for any melanoma detection assay to include *TERT* promoter mutations in order to maximize detection rates in *BRAF/NRAS* WT patients. The Guardant360 NGS ctDNA assay (Guardant Health, Redwood City, CA, USA) includes *TERT* promoter mutations (47–50), but this pan-cancer panel is not adjustable or specifically tailored for melanoma and requires an allele frequency above 0.25% to detect mutations with 100% sensitivity. We wanted to explore whether anchored multiplex PCR technology (51), which enables the enrichment of target DNA using gene specific primers located at only one end of the DNA, could concurrently detect *TERT* promoter mutations and driver oncogenes in melanoma liquid biopsies. In this proof of principle study we developed a pilot melanoma NGS panel for ctDNA analysis incorporating 15 genes and the *TERT* promoter. The performance of this custom melanoma mutation panel was evaluated in 21 stage III and IV cutaneous melanoma patient blood samples and compared directly to our previous custom melanoma panel which was based on an Ampliseq-HD workflow (25). Our data confirm that anchored multiplex PCR provided a sensitive and specific melanoma liquid biopsy assay that detects common *TERT* promoter mutations. The design of this panel can be expanded and adjusted to incorporate treatment resistance and predictive mutations and is, therefore, particularly valuable in cutaneous melanoma where most patients will ultimately relapse while on treatment with targeted or immune checkpoint therapies.

## MATERIALS AND METHODS

### Human Melanoma Samples

The fresh-frozen tissue and blood samples from melanoma patients used in the current study were obtained from the Melanoma Institute Australia biospecimen bank with written informed patient consent and institutional review board approval (Sydney Local Health District Human Research Ethics Committee, Protocol No. X15–0454 and HREC/11/RPAH/444). Healthy blood samples were obtained with written informed patient consent and institutional review board approval (Macquarie University Human Research Ethics Committee Protocol No. 52020195621941). The Oncofocus/OncoCarta panels v1.0 (Agena Bioscience, San Diego, CA, USA) or Find IT solid tumor panel (Sonic Genetics, Macquarie Park, NSW, Australia) were used for detection of melanoma-associated *BRAF*, *NRAS*, *KRAS* and *KIT* variants in tissue samples (52, 53). Immunohistochemistry to detect BRAF V600E using VE1 monoclonal antibody (Abcam, Cambridge, UK) was performed as previously described (54).

Blood (10 ml) was either collected in EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and processed within 4 h from blood draw or Cell-Free DNA collection tubes (Roche, Basel, Switzerland) and processed within 4 days from blood draw. Tubes were spun at 800 g for 15 min at room temperature. Plasma was then removed into new 15 ml tubes without disturbing the buffy coat and respun at 1600 g for 10 min at room temperature to remove cellular debris. Plasma was stored in 1-2 ml aliquots at -80°C.

### Purification of Circulating Free DNA (cfDNA) From Plasma

Plasma cfDNA was purified using the QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cfDNA was purified from to 2-5 mL of plasma. cfDNA was subsequently quantified using a Qubit dsDNA high sensitivity assay kit and a Qubit fluorometer 3 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

### Purification of DNA From Melanoma Cell Lines

Short term cultures (1-2 weeks) of melanoma cell lines were maintained in Dulbecco's Modified Eagle media supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich, St

Louis, MO, USA), 4 mM glutamine (Sigma-Aldrich, St Louis, MO, USA), and 20 mM HEPES (Sigma-Aldrich, St Louis, MO, USA), at 37°C in 5% $CO_2$. Spent medium (4 ml) was harvested 48-72 h after splitting of cells. Medium was centrifuged at 800 g for 15 min and supernatant transferred to a new tube and spun at 1600 g for 10 min. Double spun supernatant was then aliquoted into cryovials and stored at -80°C. DNA was subsequently extracted from harvested medium supernatant as described for plasma cfDNA.

## Custom Melanoma Gene Panel for Targeted NGS of cfDNA

A made-to-order melanoma gene panel consisting of individual forward and reverse primers was obtained from ArcherDX a subsidiary of Invitae (San Francisco, CA, USA). The panel covers nucleotide variants which give rise to melanoma-associated amino acid changes across 15 gene targets (42, 55–58), as well as melanoma-associated nucleotide variants in the promoter region of the *TERT* gene (36, 59) (**Table S1**).

The targeted NGS workflow was based on anchored multiplex PCR (AMP) (51). This consists of the use of anchored nested gene specific primers coupled with universal primers in two rounds of PCR amplification. NGS library preparation and sequencing workflows were according to the manufacturer's protocols (ArcherDX Liquidplex protocol for Illumina version LA090.2) with panel-specific volumes and cycling conditions according to the manufacturer's product insert (ArcherDX Liquidplex Macquarie Melanoma version LA771.1). For the second PCR reaction the number of cycles was reduced from 20 to 19.

Library QC and sequencing was performed by the Australian Genome Research Facility (AGRF) Sydney node (Westmead, NSW, Australia). Library quality was assessed using a high sensitivity D1000 screen tape on an Agilent Tapestation 2200 (Agilent, Santa Clara, CA, USA). Individual libraries were quantified using an NEBNext Library Quant Kit for Illumina (NEB, Ipswich, MA, USA) using a CFX384 Real-Time System (Bio-Rad, Hercules, CA, USA). Library concentrations were calculated using a size of 150 bp and subsequently pooled to 4 nM. The final library concentration used for sequencing was 18 pM and included 10% PhiX. Sequencing was performed using a MiSeq reagent kit V3 (600 cycle) (Illumina, San Diego California) run as 300 cycle (150 bp PE) on a MiSeq instrument (Illumina, San Diego California).

Analysis of fastq sequencing files was performed using Archer suite analysis version 6.2.7 at https://analysis.archerdx.com according to the manufacturer's protocol (Archer Analysis 6.0 User Manual). The detection of background sequencing noise was performed using a normal data set consisting of cfDNA from three healthy controls. For SNP/indel detection the thresholds included: AO ≥ 5; UAO ≥ 3; gnomAD AF ≤ 5%; AF ≥ 95MDAF. Rather than setting an average static background (cutoff) noise profile across a panel Archer analysis establishes a position-specific background noise profile for a panel, based on a normal data set, and this information is used to determine the 95MDAF for every potential variant covered by the panel. Thus, 95MDAF is a function of the sequencing coverage of the variant position and the likelihood of that variant appearing as a result of noise. This position-specific value will therefore differ between variants within the same sample and for the same variant across different samples depending on the sequencing coverage (Invitae 95MDAF technical note APM059.A).

- Alternate observation (AO)=Total number of reads that support the alternate allele.
- Unique alternate observation (UAO)=Total number of unique start sites represented by all the alternate reads that intersect the variant.
- Allele fraction (AF)= Reads that support the alternative allele (AO/DP).
- Depth (DP)= The total high quality unique molecule depth covering the variant.
- gnomAD AF =The frequency of the allele called at this locus, from gnomAD global population (60) (https://gnomad.broadinstitute.org).
- 50 Minimal detectable allele fraction (50MDAF)= The AF at which we would consider a variant significant (i.e., above the background noise) given the provided Normal Data Set and taking all consensus reads into account. If the true AF in the sample is at least this, and this identical experiment were run multiple times, 50% of the time there would be sufficient signal to capture this variant.
- 95 Minimal detectable allele fraction (95MDAF) = The AF at which we would consider a variant significant (i.e., above the background noise) given the provided Normal Data Set and taking all consensus reads into account. If the true AF in the sample is at least this, and this identical experiment were run multiple times, 95% of the time there would be sufficient signal to capture this variant.
- Read or unique fragment = Deduplicated consensus read (i.e. molecular bin) having the same unique molecular barcode.

## ddPCR Analysis

The copy number of ctDNA per µl and MAF was determined using the QX200 ddPCR (Bio-Rad, Hercules, CA, USA) as previously described (7). The amount of input DNA template varied for plasma cfDNA while for melanoma cell lines 5 ng of DNA was used for ddPCR. Cancer-associated BRAF V600E and NRAS Q61K/L/R mutations were detected using ddPCR mutation detection assays (Bio-Rad, Hercules, CA, USA). *TERT* promoter mutations -124 C>T and -146 C>T were identified using ddPCR expert design assays dHsaEXD20945488 (*TERT* C228T_88) and dHsaEXD85215261 (*TERT* C250T_88) (61) (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The *TERT* promoter assays were optimized by inclusion of 200 µM 7-deaza-dGTP (New England Biolabs, Ipswich, MA, USA), as previously described (25, 62). The DNA copy number/µl for mutant and wild-type circulating DNA species was determined with QuantaSoft software version 1.7.4 (Bio-Rad, Hercules, CA, USA) using a manual threshold setting. The minimum number of positive droplets for calling a mutation was set at two.

## Statistical Analysis

Pearson correlation coefficient analysis and generation of violin plots was performed using Graphpad Prism version 9.1.2.

# RESULTS

## Cohort and Sample Characteristics

A total of 19 cutaneous melanoma patients and three healthy controls were recruited between August 2015 and May 2021. Of the 19 melanoma patients, 2 had blood samples collected at 2 timepoints making a total of 21 melanoma samples for NGS analysis. Of the melanoma patients, 4/19 (21%) had stage III melanoma with a median age of 73 years (63–83) and 15/19 (79%) had stage IV melanoma with a median age of 65 years (30–88) (**Table 1**). The median age of the healthy control cohort was 39 years (range 29-54) and consisted of 2 females and one male.

Tissue mutation analysis was available for all patients: 8/19 (42%) had a BRAF V600 mutation, 1/19 (5%) had a BRAF non-V600 mutation, 6/19 (32%) had an NRAS Q61 mutation and 4/19 (21%) patients were BRAF/NRAS wild type (**Table 1**).

## Evaluation of the Performance of the Custom Melanoma ctDNA Panel

Based on the 15 gene targets (**Table S1**) our custom melanoma ctDNA panel was predicted to cover 66% of cutaneous melanoma patients' mutations [skin cutaneous melanoma TCGA dataset (45, 46)], 88% of uveal melanoma patients' mutations [uveal melanoma TCGA dataset (45, 46)] and 24% of acral melanoma patients' mutations [acral melanoma TCGA dataset (45, 46)]. For cutaneous melanoma the combination of BRAF V600 and NRAS Q61 mutation targets in the custom panel was predicted to cover 60% of patients [skin cutaneous melanoma TCGA dataset (45, 46)]. Addition of *TERT* promoter mutation targets in the custom panel was predicted to further increase coverage by ~15% in those cutaneous melanoma patients that are NRAS or BRAF WT (33, 38). In contrast for uveal melanoma *TERT* promoter mutations are extremely rare (63, 84). For this reason, we focused only on cutaneous melanoma when testing the panel. The custom panel design included several gene targets that were not included in our previous Thermofisher custom panel design (25) and several gene targets not included in the commercial Guardant360 pan-cancer panel (**Table S1**).

The performance of the custom melanoma ctDNA panel was initially evaluated based on unique amplification fragments generated from each forward (+) and reverse (-) gene specific primer (with the universal primer) for each of the 34 target regions (**Figure 1A**). Overall, for the 24 samples analyzed, made up of 21 melanoma patient samples and 3 healthy controls, the mean coverage (based on unique fragments) was similar for the majority of fragments (range 423-1831 unique fragments across 34 target regions). The lowest mean unique fragment count was observed for both fragments covering the *STK19* gene target (STK19- and STK19+ 423 and 478 fragments, respectively), and the *TERT* promoter primers produced 1250 and 768 unique fragments for the TERT+ and TERT- primers, respectively (**Figure 1A**).

Although each sample NGS library passed QC (as described in material and methods) and was sequenced at the same final concentration, there was variation in the median unique fragment count across the 34 amplified target regions for each sample (**Figure 1B**). This did not predict the ability to detect ctDNA mutations as the 7 mutation negative samples (7, 8, 12, 15, 17, 18, 20 and 21) had a median unique fragment count ranging from low to high (**Figure 1A**: median unique fragment count values of 479-2194). Further, this variation in the median unique fragment count was not reflected in the raw paired end reads obtained for each sample which were generally similar (median 654,127 reads) with the exception of sample 7 which had 1,0297,547 raw paired end reads (**Figure S1**). Although sample 7 had the highest paired end reads (**Figure S1**), it did not have the highest mean unique fragment count (**Figure 1B**). Variation in sample performance was not due to the quantity of cfDNA template. For 20 of 24 samples the input was 20 ng while for the remaining samples 10, 20, 22 and 24 the input range was 11.9-15.9 ng. Those samples with the lowest cfDNA input did not yield consistently low median unique fragment counts (**Figure 1B**). Variations in the size distribution of ctDNA may account for variation in sequencing performance. It has been shown that enrichment of ctDNA in the size range 90-150 bp from patients with melanoma improves ctDNA detection by sequencing (64).

## Identification of Melanoma BRAF and NRAS Mutations Using the Custom Melanoma ctDNA Panel

The ArcherDX custom melanoma ctDNA panel detected 13/17 (76%) patients with *BRAF*- or *NRAS*-mutant melanoma. This included 12/14 (86%) stage IV and 1/3 (33%) stage III melanoma patients (**Figure 2**). Liquid biopsies revealed an additional NRAS Q61R-driver mutation in the BRAF V600-mutant samples 4 and 59 from the same patient, and both NRAS mutations were at less than 1% allele frequency compared to the ~30% BRAF mutation frequency (**Tables S2**, **S3**). Initially, only tissue immunohistochemistry was used to detect BRAF V600E (65) in these samples. Subsequently, ddPCR analysis of a patient derived melanoma cell line (corresponding to a timepoint 12 months after liquid biopsy samples 4 and 9) confirmed the BRAF V600E mutation but not the NRAS Q61R mutation (ddPCR data not shown). The negative NRAS Q61R signal is likely due to the time point difference from liquid biopsy samples and the fact this was a selected cell population highlighting the subclonal nature of the NRAS mutation. Although, oncogenic *BRAF* and *NRAS* mutations are usually mutually exclusive (66), they co-occur in approximately 7% of untreated melanoma and *NRAS* mutations confer resistance in 27% of $BRAF^{V600}$-mutant melanoma patients who progress on combination BRAF and MEK inhibitor therapy (67). It is worth noting that this patient (corresponding to samples 4 and 9) had prior combination dabrafenib and trametinib (combi-DT) treatment. The fact that

**TABLE 1** | Patient characteristics.

| Clinical Characteristics | Stage III patients (n = 4) | Stage IV patients (n = 15) |
|---|---|---|
| Age – median (range) | 73 (64–84) | 65 (30-88) |
| Sex – no. (%) | | |
| Male | 2 (50) | 13 (87) |
| Female | 2 (50) | 2 (13) |
| AJCC tumor stage (89) – no. (%) | | |
| M1a or M1b | NA | 5 (33) |
| M1c | NA | 5 (33) |
| M1d | NA | 5 (33) |
| Mutation – no. (%) | | |
| BRAF V600 | 0 (0) | 8 (53) |
| BRAF non-V600 | 0 (0) | 1 (7) |
| NRAS | 2 (50) | 4 (27) |
| BRAF/NRAS WT | 2 (50) | 2 (13) |
| Timing of blood draw* | (n = 5) | (n = 16) |
| Pre (treatment naïve) or at time of treatment progression | 4 (80) | 13 (81) |
| EDT (within 3 weeks) | 1 (20) | 3 (19) |

**One patient from stage III cohort and 1 patient from stage IV cohort had 2 samples. EDT patients did not respond (no complete or partial responders) to treatment.*
*AJCC, American Joint Committee on Cancer; EDT, early during therapy; NA, not applicable.*

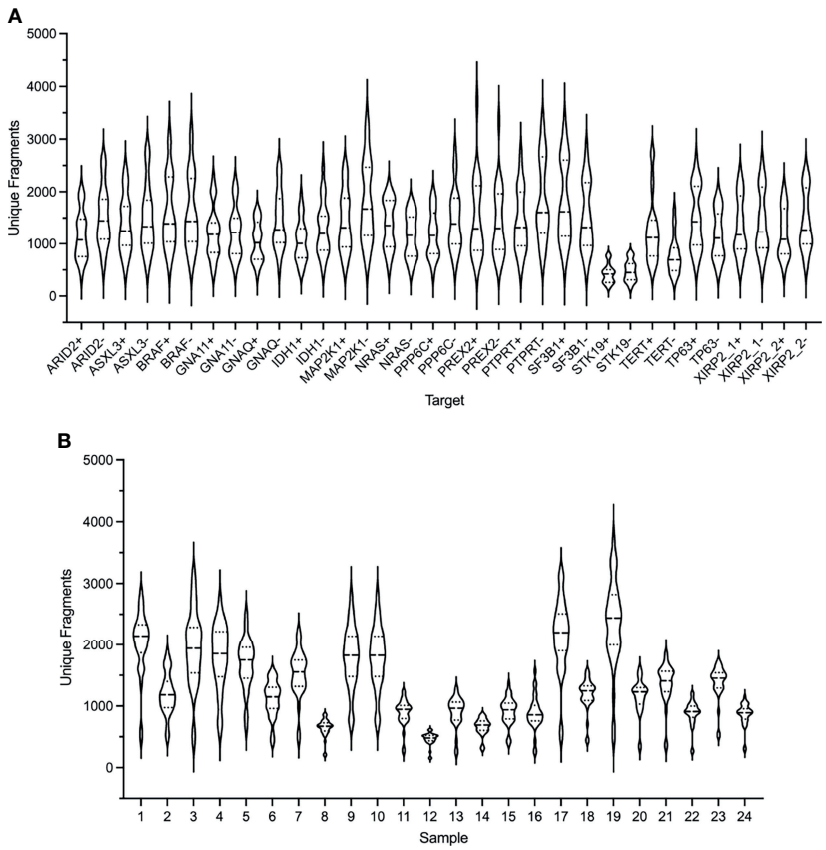


**FIGURE 1** | Performance of the ArcherDX custom melanoma ctDNA panel. **(A)** Distribution of unique fragments for each gene specific target in the custom melanoma panel based on sequencing of 24 samples. For each gene target (see **Table S1** for targets) both + and – DNA strand generated fragments are shown. **(B)** Distribution of unique fragments obtained for each sample based on the 34 targets shown in **(A)**. Samples 1-21 are melanoma patients and 22-24 are healthy controls. Next generation sequencing (NGS) libraries were generated using an ArcherDX Liquidplex NGS workflow followed by Illumina MiSeq sequencing. Unique fragments were defined as deduplicated consensus reads having the same unique molecular barcode. Violin plots show median and SD.

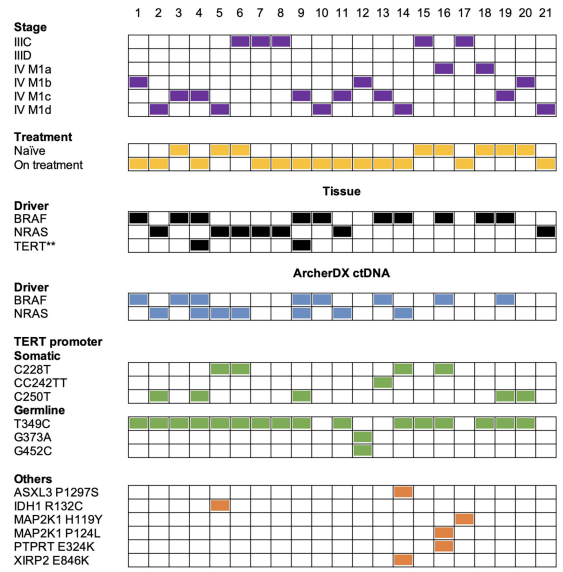


**FIGURE 2** | Summary of the melanoma mutation profile identified across the 21 melanoma patient samples from a cohort of 19 melanoma patients. Comparison of melanoma stage (purple boxes), treatment (yellow boxes), tissue driver mutations (black boxes) versus mutations detected with the ArcherDX custom melanoma ctDNA panel including driver mutations (blue boxes), *TERT* promoter somatic mutations or germline variations (green boxes) and other cancer-associated mutations (orange boxes). Numbers represent sample number. For further details on specific gene variations identified and patient details refer to **Table S2**. Samples 4 and 9 are derived from a single patient at two time points. Samples 7 and 8 are derived from a single patient at two time points. **Mutation data from a cell line derived from patient tissue collected 12 months after liquid biopsy samples.

two liquid biopsy samples collected from the same patient at different times displayed identical BRAF/NRAS mutation profiles provides confidence that these mutation data are accurate.

Mutations detected in plasma and tissue were concordant in 16/21 patients (76%) and included 12 *BRAF/NRAS* mutation positive and 4 *BRAF/NRAS* mutation negative patients (**Figure 2**). Five samples (samples 7, 8, 14, 18, 21) had a detectable driver mutation in the tissue that was not identified in the liquid biopsy (**Table S2**). We confirmed in samples 7, 8 and 18 that the driver mutation could not be detected in ctDNA using single molecule ddPCR (**Table S3**). Analysis of an NRAS Q61R-mutant NM47 melanoma cell line (68) confirmed that the Q61R ddPCR signal for patient-matched stage III samples 7 and 8 consists of low droplet counts with low intensity (**Figure S3**). Thus, we cannot confidently detect the NRAS Q61R mutation in these two samples by ddPCR or sequencing. ddPCR was unable to detect a BRAF V600E mutation in sample 18 (**Table S3**). Sample 14 had a BRAF G469E mutation which was not covered by our custom ArcherDX panel (**Table S1**). BRAF G469E is a deactivating BRAF mutation that promotes melanomagenesis through oncogenic NRAS (45, 46, 69). This patient has the less common NRAS G60V detected in the liquid biopsy (**Table S2**). This NRAS mutation [TCGA dataset (45, 46)] is predicted to be a driver mutation (COSM4606360). For sample 21 no remaining ctDNA or plasma was available for ddPCR.

## Identification of Melanoma TERT Promoter Mutations Using the Custom Melanoma ctDNA Panel

In contrast to our previous NGS study using a Thermofisher custom melanoma ctDNA panel (25), the ArcherDX custom melanoma ctDNA panel detected *TERT* promoter mutations in ctDNA. Cancer-associated *TERT* promoter mutations (C228T, CC242TT and C250T) (36, 59) were identified in 10/21 (48%) of melanoma samples (**Figure 2**). Of these 5/10 (50%) were C250T, 4/10 (40%) were C228T and 1/10 (10%) was CC242TT. For patient ID 4, corresponding to samples 4 and 9, we did confirm *TERT* promoter mutation C250T in a cell line derived from tissue of the same patient collected 12 months after liquid biopsy using ddPCR (**Figure 2**; ddPCR data not shown). This distribution of *TERT* promoter mutations aligns with previous studies on melanoma (33, 38). Co-occurrence of *TERT* promoter mutations with either BRAF or NRAS mutations was found in 9/10 (90%) of the samples. The detection of a *TERT* C250T mutation in sample 20 (**Figure 2**), which was BRAF and NRAS mutation negative based on both the tissue panel and custom panel (**Table S2**), increased the overall detection rate for the custom panel from 13/21 (62%) to 14/21 (67%) (based on *BRAF/NRAS/TERT* promoter).

Although unique fragment coverage for *TERT* promoter centered around cancer-associated *TERT* promoter mutations, the nature of anchored multiplex PCR did result in coverage beyond the target region albeit with lower read depth (**Figure S2**). Therefore we were able to detect a number of previously described germline *TERT* promoter variations (70) including T349C (rs2853669), G373A (rs35226131) and G452C (rs35161420) at MAFs ranging from 38-99% (**Figure 2** and **Table S2**). None of these variations were present in the 3 healthy controls (data not shown). Of these previously described germline variations, T349C was found in 14/21 (67%) patients while G373A and G452C co-occurred in 1/21 (5%) patients (**Figure 2**). A previous study found *TERT* T349C to be present in 52% of melanoma cell lines (43). The high frequency of T349C also aligns with previous studies on other cancers (70–72). All the *TERT* promoter mutations C228T and C250T and 14/17 (82%) *BRAF/NRAS* mutations co-occurred with the germline *TERT* promoter variant T349C (**Figure 2**). There was no clear relationship between either melanoma stage or *BRAF/NRAS* mutation status and the presence of *TERT* promoter variant T349C (**Figure 2**).

## Identification of Other Melanoma Cancer-Associated Mutations Using the Custom Melanoma ctDNA Panel

Several other cancer-associated mutations were identified in 4/21 (19%) patients using the ArcherDX custom melanoma ctDNA panel (**Figure 2**). Of the BRAF and NRAS mutation negative samples, the custom panel was able to detect a MAP2K1 H119Y mutation in sample 17 (**Figure 2**). The patient, who was BRAF

V600 WT, had been treated for 1 week with pembrolizumab and subsequently maintained persistently detectable disease. This mutation has been recently proposed to be a non-hotspot *MAP2K1* mutation which activates the ERK pathway (73) and therefore could be targeted with an ERK inhibitor (74). The remaining mutations did not increase detection coverage of the custom panel as they all co-occurred with either BRAF or NRAS driver mutations (**Figure 2**). Of note, PTPRT E324K, along with MAP2K1 P124L, was detected in treatment naïve sample 16 which also harbored a BRAF V600K mutation (**Figure 2**). In melanoma, mutations in PTPRT, such as E324K, which create neoepitopes, may be associated with better outcomes for patients on immunotherapy (75). This may have been an option for this patient who subsequently had a partial response to combination BRAF/MEK inhibition with the presence of MAP2K1 P124L presumably contributing to resistance (76, 77). For sample 14, ASXL3 P1297S and XIRP2 E846K were detected in an NRAS G60V background which was also BRAF G469E (based on tissue biopsy) (**Figure 2** and **Table S2**). Given the MAF of ASXL3 P1297S was 55.88% and this mutation is not a previously identified melanoma-associated mutation (45, 46), suggests that it may be a germline polymorphism or a result of clonal hematopoiesis, which has been noted for mutations in the related protein ASXL1 (78). Sample 5 had an NRAS Q61 and IDH1 R132 mutation in the liquid biopsy and the co-occurrence of these mutations (**Figure 2**) is significant ($p<0.001$) based on the skin cutaneous melanoma TCGA database analysis of mutual exclusivity (45, 46).

## Sensitivity of the Custom Melanoma ctDNA Panel

The cutoff for calling a mutation, based on the 95% confidence interval for the normal samples (95MDAF as defined in material and methods), of the custom melanoma ctDNA panel was inversely proportional to read depth but did approach a saturation point beyond which increasing read depth did not lead to a lower 95MDAF value (**Figure 3**). This 95MDAF value was ~0.3% for BRAF, 0.26% for *TERT* promoter mutations and 0.2% for NRAS mutations (**Figure 3**). The custom panel was also able to detect a MAP2K1 H119Y mutation with an 95MDAF of 0.18% (**Table S2**). In addition, several cancer-associated mutations were noted whereby the MAF was <95MDAF but still >50MDAF as defined in material and methods (**Table S2**). For these mutations further validation would be required. To demonstrate the accuracy of these low MAF cutoff values does ultimately require running a dilution series of samples for each mutation consisting of known MAFs.

## Validation of the Custom Melanoma ctDNA Panel

All of BRAF V600E/K or NRAS Q61K/L/R driver mutations identified using the ArcherDX custom melanoma ctDNA panel

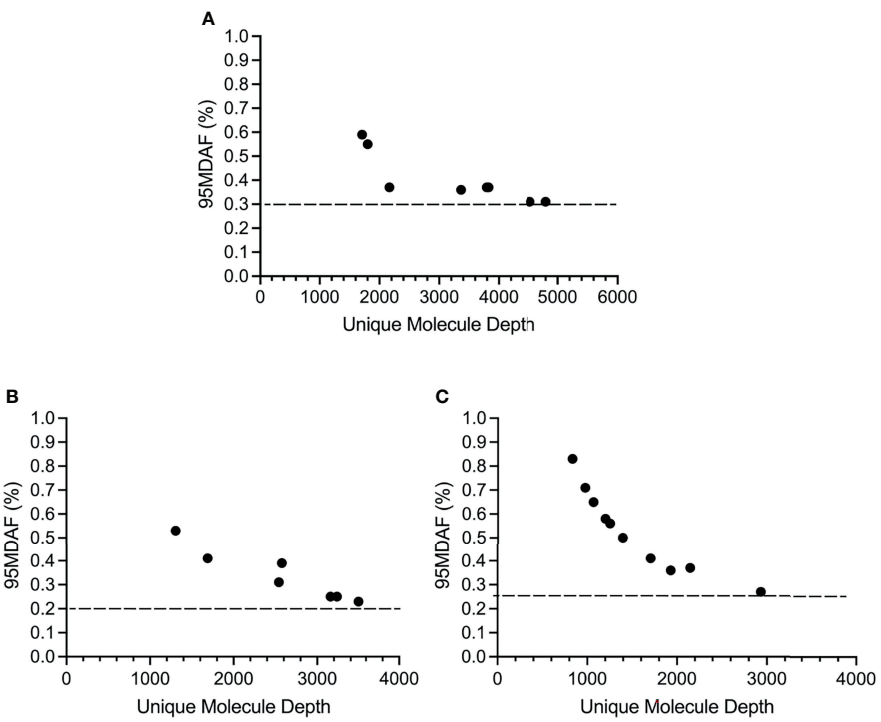


**FIGURE 3** | Sensitivity of the custom melanoma ctDNA panel based on BRAF, NRAS or *TERT* promoter mutations identified across the melanoma cohort. Each circle corresponds to the 95 minimal detectable allele fraction (95MDAF) values and unique molecule depth (unique fragments covering the specified region) for a single sample. **(A)** BRAF V600 mutations. Data derived from samples 1, 3, 4, 9, 10, 13, 16, 19. **(B)** NRAS G60/Q61 mutations. Data derived from samples 2, 4, 5, 6, 9, 11, 14. **(C)** TERT 228-250 promoter mutations. Data derived from samples 2, 4, 5, 6, 9, 13, 14, 16, 19, 20.

and tissue biopsy were also identified using either ddPCR or a Thermofisher custom melanoma ctDNA panel (25) (**Table S2**). Furthermore, there was significant correlation in the MAF for each of these identified mutations when comparing the ArcherDX custom panel to these other liquid biopsy assays (**Figure 4A**). All of the *TERT* promoter C228T and C250T mutations identified with the custom panel were confirmed using ddPCR and showed significant correlation based on MAF (**Figure 4B**).

## DISCUSSION

In this study, we have developed a custom melanoma NGS panel for detection of ctDNA. This panel was based on multiplex anchored PCR (51) from ArcherDX in contrast to previous studies which used either Thermofisher Ampliseq HD (25, 27), Qiagen QIAseq (24) or Illumina TruSeq Nano (28) workflows. An additional custom melanoma panel based on mass spectrometry detection has also been reported (29). None of

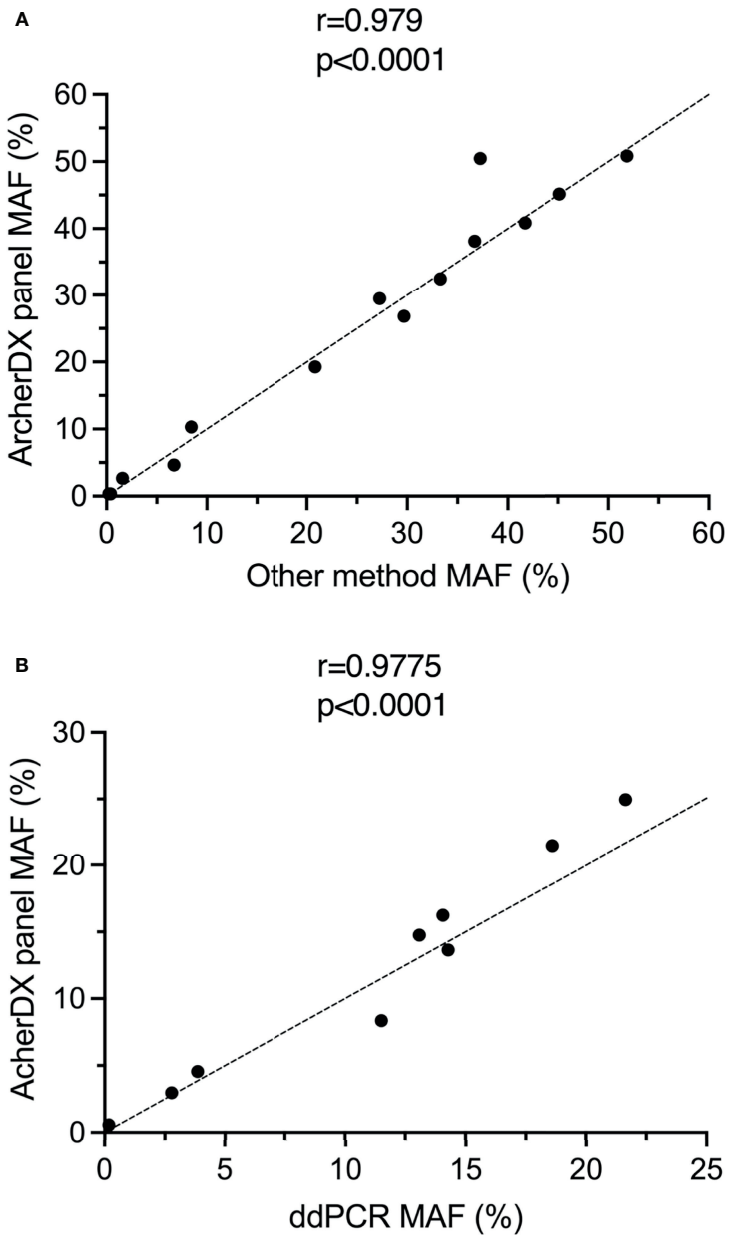


**FIGURE 4** | Validation of the ArcherDX custom melanoma ctDNA panel. **(A)** Correlation of driver mutant allele frequency (MAF) determined by the ArcherDX custom melanoma ctDNA panel versus other liquid biopsy analysis [ddPCR or Thermofisher custom melanoma ctDNA panel (25)]. **(B)** Correlation of *TERT* promoter MAF determined by the ArcherDX custom melanoma ctDNA panel versus ddPCR. For ddPCR data see **Table S3**. Pearson correlation coefficient analysis was performed.

these previously reported custom panels have successfully detected *TERT* promoter mutations, and our previous Thermofisher NGS panel yielded consistently low sequencing depth for the *TERT* promoter amplicons presumably due to the high GC content (>80%) (25). Given that *TERT* is one of the most frequently mutated genes in melanoma (42), the primary goal of our NGS panel design was to reliably detect the three common *TERT* promoter mutations in ctDNA.

The design of the current custom melanoma ctDNA panel was limited to key melanoma-associated gene mutations, but still had a theoretical coverage of ~81% of skin cutaneous melanoma [66% based on skin cutaneous melanoma TCGA database (45, 46) plus (15-60%) triple wild type melanoma with *TERT* promoter mutations (33, 38–40, 43, 44)]. Mutually exclusive somatic *TERT* promoter mutations, C250T, C228T and CC242TT, were found in 48% (10/21) of the melanoma cohort and increased the overall ctDNA detection rate from 67%, based on detection of driver (*NRAS* or *BRAF*) or cancer-associated mutations, to 71%. The limitation of the current study was that only four triple wild type samples were included. The custom melanoma panel detected only 50% of these four cases (based on detection of a *TERT* C250T mutation and a *MAP2K1* H119Y mutation respectively). Based on a *TERT* mutation frequency of 15-60% in triple wild type melanoma (33, 38–40, 43, 44) the *TERT* mutation detection rate of 25% of our wild type melanoma patients using this panel falls within the frequency range. A larger study incorporating many more triple wild-type melanoma samples is needed to accurately determine the detection sensitivity of this pilot ctDNA panel. We expect that analysis of a larger randomly selected or cross-sectional cohort would find more frequent *TERT* promoter mutations in the absence of *BRAF* or *NRAS* mutations, as previously reported (33, 38, 39). It is worth noting, however, that *TERT* promoter mutations may be subclonal and therefore under-represented in ctDNA relative to other driver mutations (24).

The overall detection rate of 71% in the current study was less than our previous custom ctDNA panel detection rate of 74% primarily due to a larger number of genes covered in our previous study (25). This was the case with lack of detection of BRAF G469E in one patient as this infrequent melanoma mutation (0.1% incidence) was not covered in the custom panel design. On the other hand, the custom panel did detect a rare NRAS G60V mutation in the same patient which was not detected by the tissue panel. This may possibly be due to the fact it is not a listed variant for tissue panels and therefore not called during analysis. A detectability of 20% in the stage III cohort was low although this was only based on five patient samples.

The ability to detect somatic *TERT* promoter mutations co-occurring with *BRAF* or *NRAS* mutations is also valuable given that a TERT-mutation positive genetic profile is associated with a worse prognosis for melanoma patients (30, 38, 79). Further reports suggest that *TERT* promoter mutations may be predictive of improved response to immunotherapy (80) and a poorer response to BRAF/MEK inhibition (35). Given our small cohort size and the fact that the majority of the patients were non-responders to immunotherapy, we were unable to conclude whether detection of *TERT* promoter mutations does in fact have any prognostic or predictive value.

We also detected the previously described germline single nucleotide polymorphisms (SNPs) in the TERT promoter T349C, G373A, G452C (70) in 81% of our melanoma cohort at a MAF ranging from 38 to 99%. The most frequent *TERT* SNP (T349C; rs2853669) was found in 76% of our melanoma cohort. This *TERT* SNP has been identified in a range of other cancers and may act to disrupt a pre-existing ETS binding site in the *TERT* promoter (81). Nevertheless, its prognostic role remains controversial with contrasting reports on its influence on *TERT* expression and differing conclusions on its prognostic value (43, 70–72, 82, 83, 85–88). In melanoma, *TERT* T349C has been reported to modify the effects of somatic *TERT* promoter mutations leading to increased survival in melanoma (40) and this may be mediated through a lengthening of telomeres (43). We found no obvious association of this variation either with somatic *TERT* mutation or patient outcomes in our melanoma cohort.

In addition to successful detection of *TERT* promoter mutations our current custom melanoma panel performed favorably, based on tissue concordance (76%), 95MDAF (0.2%) and significant correlation with ddPCR, when compared to previously published custom panels (24, 25, 27–29). Future studies will incorporate our findings from previous (25) and current panel designs to produce an optimized melanoma custom ctDNA panel with detection rates of at least 90% in stage IV cutaneous melanoma patients. An optimized melanoma ctDNA (50 gene targets), which will increase the theoretical coverage will be useful for monitoring residual disease in stage III patients after resection and therapy and for longitudinal monitoring of progression in stage IV melanoma patients. In both cases ctDNA mutations identified pre-treatment will be monitored longitudinally using targeted approaches such as ddPCR. To improve ctDNA detection sensitivity ctDNA mutation detection may also be complemented with ctDNA methylation analysis. Ultimately establishing if our optimized melanoma panel truly reaches the theoretical coverage will depend on analysis of a larger cohort consisting of 100-200 patients.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/, PRJNA798432, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA798432.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sydney Local Health District Human Research Ethics Committee, Protocol No. X15–0454 and HREC/11/RPAH/444. Macquarie University Human Research Ethics Committee Protocol No. 52020195621941. The patients/

participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Conceptualization, RD, JL, and HR. Methodology, RD and HR. Formal analysis, RD, JL, AS, and HR. Investigation, RD, JL, and AS. Resources, AM, MC, GL, RPMS, JS, and RAS. Writing—original draft preparation, RD and HR. Writing—review and editing, RD, JL, AS, AM, MC, GL, RPMS, JS, RAS, and HR. Visualization, RD, JL, and HR. Supervision, HR. Project administration, HR. Funding acquisition, HR. All authors contributed to the article and approved the submitted version.

## FUNDING

RD was supported in part by a donation to Melanoma Institute Australia from the CLEARbridge Foundation. This work was also supported in part by the National Health and Medical Research Council (APP1093017 and APP1128951). HR, RAS, and GL are supported by National Health and Medical Research Council Research Fellowships (APP1104503, APP1141295 and APP1119059). GL is also supported by the Medical Foundation of The University of Sydney and Melanoma Institute Australia. AMM is supported by a Cancer Institute NSW Fellowship, Nicholas and Helen Moore and Melanoma Institute Australia. JS and RPMS are supported by Melanoma Institute Australia. Support from colleagues at Melanoma Institute Australia, Royal Prince Alfred Hospital and Westmead Hospital is also gratefully acknowledged. Support from The Ainsworth Foundation is also gratefully acknowledged.

## ACKNOWLEDGMENTS

Biobank and sample collection support from Hazel Burke, Maria Gonzalez, Rebecca Velickovic, and Rebecca Kasalo (Melanoma Institute Australia) as well as Gilles Guillemin and Benjamin Heng (Macquarie University) is much appreciated. ArcherDX workflow and analysis support from Hamilton Fraval, Zena Kassir and Woei Loon is also much appreciated.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.820510/full#supplementary-material

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**Conflict of Interest:** GL receives consultant service fees from Amgen, BMS, Array, Pierre-Fabre, Novartis, Merck Sharp & Dohme (MSD), Qbiotics, Roche and Sandoz. AM is an advisory board member for BMS, MSD, Novartis, Roche, Pierre Fabre and Qbiotics. MC is an advisory board member for MSD, BMS, Novartis, Pierre-Fabre, Roche and Amgen. RAS has received fees for professional services from Evaxion, Provectus Biopharmaceuticals Australia, Qbiotics Group Limited, Novartis Pharma AG, MSD Sharp & Dohme (Australia), NeraCare, AMGEN Inc., Bristol-Myers Squibb, Novartis Pharmaceuticals Australia Pty

Limited, Myriad Genetics GmbH, GlaxoSmithKline Australia. RPMS has received honoraria for advisory board participation from MSD, Novartis and Qbiotics and speaking honoraria from BMS.

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frontiers | Frontiers in Oncology

ORIGINAL RESEARCH
published: 05 July 2022
doi: 10.3389/fonc.2022.901547

# Circulating Tumor DNA Characteristics Based on Next Generation Sequencing and Its Correlation With Clinical Parameters in Patients With Lymphoma

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**Specialty section:**
*This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology*

**Received:** *22 March 2022*
**Accepted:** *13 May 2022*
**Published:** *05 July 2022*

**Citation:**
*Wu X-B, Hou S-L, Zhang Q-H, Jia N, Hou M and Shui W (2022) Circulating Tumor DNA Characteristics Based on Next Generation Sequencing and Its Correlation With Clinical Parameters in Patients With Lymphoma. Front. Oncol. 12:901547. doi: 10.3389/fonc.2022.901547*

**Background:** Lymphoma is a heterogeneous group of tumors in terms of morphological subtypes, molecular alterations, and management. However, data on circulating tumor DNA (ctDNA) mutated genes are limited. The purpose of this study was to investigate the features of the ctDNA mutated genes, the prognosis, and the association between the ctDNA mutated genes and the clinical parameters in lymphoma.

**Methods:** Differences in the ctDNA between the mutated genes and the prognosis of 59 patients with Hodgkin's lymphoma (HL) (10.2%), germinal center B-cell–like lymphoma (GCB) (28.8%), nongerminal center B-cell–like lymphoma (non-GCB) (50.8%), and marginal zone lymphoma (MZL) (10.2%) were analyzed by next generation sequencing (NGS) targeting 121 lymphoma-relevant genes.

**Results:** Genetic alterations were identified in the ctDNA samples with a median of 6 variants per sample. The genetic variation of the ctDNA in the plasma was found to be significantly correlated with the clinical indices in lymphoma. The genetic heterogeneity of different lymphoma subtypes was clearly observed in the ctDNAs from HL, GCB, non-GCB, and MZL, confirming that distinct molecular mechanisms are involved in the pathogenesis of different lymphomas.

**Conclusion:** Our findings suggest that NGS-based ctDNA mutation analysis reveals genetic heterogeneity across lymphoma subtypes, with potential implications for discovering therapeutic targets, exploring genomic evolution, and developing risk-adaptive therapies.

**Keywords: lymphoma, tumor heterogeneity, circulating tumor DNA (ctDNA), next-generation sequencing (NGS), gene mutation, prognosis**

## INTRODUCTION

Lymphoma is a malignant tumor that originates from the lymphopoietic system and is the most common hematologic malignancy in the world. It is divided into Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). Lymphoma is a heterogeneous group of tumors in terms of morphological subtypes, molecular alterations, and management, involving a complex diagnosis and management, and different prognoses. There are significant differences in the response of these tumors to standard treatment strategies. Therefore, access to tumor components and genetic material is essential for diagnosis, management, and the selection of targeted therapies.

The prognosis of classical HL has improved with the advancement of novel therapeutic strategies, resulting in a high cure rate (1), and current genomic technologies have also greatly improved the disease classification and prognostication of major subtypes of B-cell lymphomas (2). However, critical clinical needs remain unmet. The estimated 5-year overall survival (OS) was 96.0%–99.4% in the early stages of HL, using the European Organization for Research and Treatment of Cancer staging criteria (3), but the 5-year OS ranges from 42% to 81% only in the advanced-stage disease (4). The combination of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) cures approximately 65% of patients with diffuse large B-cell lymphoma (DLBCL). Patients who do not respond to R-CHOP therapy or who experience relapse are treated with a second-line therapy. Long-term remission occurs in 20%–30% of patients but at the cost of high toxicity and treatment-related mortality (5). Therefore, understanding the mechanisms involved and identifying predictive biomarkers is essential.

Tissue biopsy is a traditional method for detecting the molecular features of tumors. However, its limitations are its invasive nature and the difficulty of obtaining serial samples in clinical practice. Given the profound intra-tumor heterogeneity (6, 7), a single-site biopsy is highly unlikely to capture the entire genomic complexity of a tumor. In fact, different regions of the same tumor may show different genetic maps, while biopsies from different parts of the tumor may miss mutations in subclones inhabiting distant sites. Liquid biopsies are based on the analysis of circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), or tumor-derived extracellular vesicles that have been shed from tumors and their metastatic sites into the blood (8). Since ctDNA is derived from tumor cells, it contains tumor-derived genetic alterations that can reflect the molecular heterogeneity of multiple disease sites (9). In the management of lymphoma, genotyping of ctDNA has been successfully integrated into clinical work (10, 11). Next-generation sequencing (NGS) technology has become a promising method for ctDNA mutation profiling due to its high throughput, better sensitivity, and specificity (12).

We analyzed the mutation profiles of different lymphoma subtypes [including HL and B cell non-Hodgkin's lymphoma (B-NHL)] using patients' ctDNA and tumor genomic DNA (gDNA). We targeted 121 related genes by NGS to explore the clinical features of ctDNA mutation profiling in lymphomas and reveal the genetic heterogeneity of different subtypes of lymphoma, with the aim of facilitating prognosis predictions and treatment decisions.

**Abbreviations:** HL, Hodgkin's lymphoma; NHL, non-Hodgkin's lymphoma; cHL, classical Hodgkin's lymphoma; OS, overall survival; DLBCL, diffuse large B-cell lymphoma; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; NGS, Next-generation sequencing; GCB, germinal center B-cell-like lymphoma; non-GCB, non-germinal center B-cell-like lymphoma; MZL, marginal zone lymphoma; gDNA, Tumor genomic DNA; SNVs, Single nucleotide variants; VCF, Variant Call Format; IGV, Integrative Genomics Viewer; MAFs, mutant allele frequencies; PTL, primary testicular lymphoma; NSCLC, non-small cell lung cancer.

## MATERIALS AND METHODS

### Study Design

From 60 patients with lymphoma who enrolled in the program, 59 patients were included in this retrospective study according to their pathology type. The clinical and follow-up data were collected and the association between them was analyzed. The pathology types included HL (n = 6), germinal center B-cell–like lymphoma (GCB) (n = 17), nongerminal center B-cell–like lymphoma (non-GCB) (n = 30), and marginal zone lymphoma (MZL) (n = 6). The patients were diagnosed with lymphoma between 2019 and 2021 at the Shanxi Bethune Hospital (Taiyuan, China). Of the 59 patients, 21 were aged 65 years or older, the median age was 60 years old, and 26 were male. The exclusion criteria were: (1) patients who have already started any treatment (including steroids) before signing informed consent; (2) patients with contraindications to positron emission tomography; (3) patients who were HIV-positive; (4) patients with hepatitis B or C; (5) pregnant women. All treatments were performed in accordance with the Declaration of Helsinki. The ethics committee of Shanxi Bethune Hospital approved this study. All patients gave informed consent for specimen collection, clinical data collection, and biomarker analysis.

Of the patients, 49.1% had a good performance status (Eastern Cooperative Oncology Group [ECOG] score 0 or 1), most patients (66.1%) presented extranodal involvement, and a minority of patients (30.5%) presented B symptoms. Most of the patients (55.9%) were in Ann Arbor Stage IV. Patients' demographic and clinical characteristics are summarized in **Table 1**.

### Sample Collection and Circulating Tumor DNA Extraction Processing

Plasma samples were collected at baseline. For each patient, 5–10 ml peripheral blood samples were collected within 24 h in ethylenediaminetetra–acetic acid-coated tubes (BD Biosciences). These were centrifuged for 10 min at 3500 rpm at 4°C within 2 h of collection and stored at −80°C. Cell-free DNA (cfDNA) was extracted from 2 ml plasma using the AVENIO cfDNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and quantified with the Qubit™ dsDNA High Sensitivity Kit (ThermoFisher). Enrichment of the characteristic mononucleosomal fragment peak (160–200 bp) and the absence of contaminating high molecular weight genomic DNA (13, 14) were verified using the Bioanalyzer 2100 High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA).

**TABLE 1** | Baseline characteristics of all patients.

| Variables | N (%) |
|---|---|
| Age (years) | |
| Median | 60 |
| Range | 24-86 |
| Gender | |
| Male | 26 (44.1) |
| Female | 33 (55.9) |
| Pathological diagnosis | |
| HL | 6 (10.2) |
| DLBCL (GCB) | 17 (28.8) |
| DLBCL (non-GCB) | 30 (50.8) |
| MZL | 6 (10.2) |
| Ann Arbor Stage | |
| I | 11 (18.6) |
| II | 7 (11.9) |
| III | 9 (15.3) |
| IV | 32 (54.2) |
| IPI/IPS | |
| <2 | 10 (16.9) |
| 2-4 | 29 (49.2) |
| >4 | 20 (33.9) |
| ECOG | |
| 0 | 6 (10.2) |
| 1 | 23 (38.9) |
| 2 | 18 (30.5) |
| 3 | 9 (15.3) |
| 4 | 3 (5.1) |
| B symptoms | |
| Present | 18 (30.5) |
| Absent | 41 (69.5) |
| Extranodal involvement | |
| With | 39 (66.1) |
| Without | 20 (33.9) |
| Complications | |
| With | 38 (64.4) |
| Without | 21 (35.6) |
| Ki-67 | |
| <10% | 3 (5.1) |
| 10%-50% | 7 (11.9) |
| >50% | 49 (83.1) |

The gDNA was isolated from formalin-fixed paraffin-embedded (FFPE) diagnostic tissue biopsies. Excess paraffin was removed from the FFPE tissue with a scalpel, and the specimens were cut to 10 μm thickness; the first 2–3 exposed and air-exposed slices were discarded, and 1–2 internal slices were reserved for DNA extraction. Sections were immediately placed in 2-ml Eppendorf centrifuge tubes, and DNA was extracted using the FlexiGene DNA kit (Qiagen, Germany) and saved at −80°C for further testing. The DNA content was determined using a NanoDrop™ 2000 ultramicroscopic spectrophotometer (ThermoFisher Scientific, USA).

## Library Construction

The fragment DNA was generated with Bioruptor® (Diagenode, Bioruptor UCD-200) following the manufacturer's instructions. Libraries were constructed using the KAPA HyperPrep DNA Library Kit (KAPA Biosystem, KK8504). Dual-indexed sequencing libraries were amplified by polymerase chain reaction (PCR) with KAPA HiFi HotStart ReadyMix (KAPA, KK2602) for 4–6 cycles, then cleaned up by purification beads (Corning, AxyPrep FragmentSelect-I Kit, 14223162). Library concentration and quality were determined by the Qubit™ 3.0 system (Invitrogen) and the Bioanalyzer 2100 (Agilent, Agilent HS DNA Reagent, 5067–4627).

## Hybrid Selection and Ultra-deep Next Generation Sequencing

The 5′-biotinylated probe solution was used as the capture probes. The probes for targeted sequencing cover exons and selected introns of 121 lymphoma-related genes. The amplified samples were purified by AMPure XP beads, quantified by quantitative PCR (KAPA) and sized on a Bioanalyzer 2100 (Agilent, Agilent HS DNA Reagent, 5067–4627). Libraries were normalized to 2.5 nM and pooled. Finally, the library was sequenced as paired 150 bp reads on an Illumina HiSeq 4000 according to the manufacturer's instructions.

## Single Nucleotide Variants and Short Insertions/Deletions Detections

Single nucleotide variants (SNVs) and short insertions/deletions (indels) were identified by VarScan 2 v2.3.9 to generate variant call format files with the minimum variant allele frequency (VAF) threshold set at 0.01 and the p-value threshold for calling variants set at 0.05, with minimum base quality = 20, minimum mapping quality = 1, the minimum coverage = 20, minimum read depth = 8, basic strand-bias filter = 1. All SNVs/indels were annotated with ANNOVAR (version 28) using the filter-based annotation based on human genome hg19 with the database dbscsnv11, and each SNV/indel was manually checked on the integrative genomics viewer (15).

## Statistics

The Chi-squared or Fisher's exact test was used to compare the samples with certain genetic alterations. A non-parametric test (Mann–Whitney) was used to determine the relationships between different molecular parameters. The correlation between mutated genes and clinical indicators was evaluated by Spearman correlation coefficient. The Kaplan–Meier method and log-rank test were used to analyze the progression-free survival (PFS) rate. The relationship between ctDNA mutations and clinical indicators was analyzed by logistic regression. The Cox proportional hazard regression model was used for univariable analyses. The SPSS™ Statistics version 25.0 software was used for all the statistical analyses, and all graphs were constructed on the Prism version 8.00 (GraphPad Software Inc, USA) and Photoshop CS5 software (Adobe Systems Software Ireland Ltd, Dublin, Ireland). A value of $P < 0.05$ was considered statistically significant.

# RESULTS

## Targeted Next Generation Sequencing Mutation Profiling of Circulating Tumor DNA and Genomic DNA From Patients With Lymphoma

Patients with DLBCL and MZL were treated with the chemotherapy regimen for R-CHOP, and patients with HL

were treated with the chemotherapy regimen for Adriamycin, bleomycin sulfate, vinblastine sulfate, and dacarbazine. The time between tissue and liquid biopsy was less than two weeks in all patients (median = 7 days, range 1–12 days).

The ctDNA and tissue biopsies were collected from all patients, and NGS analysis was performed. Patients were considered to have mutations if they had a mutation in their extracted gDNA and/or plasma ctDNA biopsies. The PFS was defined as the time from diagnosis until the date of progression, relapse, death, or the last follow-up. In the present study, variants were found in all patients. A total of 82 genes or sites were identified by genotyping of ctDNA or gDNA collected at diagnosis. In the ctDNA, 52 gene mutations were identified, of which 8 were not found in the corresponding biopsies; whereas gDNA genotyping in tissue biopsies identified 74 gene mutations, of which 30 variants were not found in the corresponding plasma samples. The maximum follow-up time was 33 months.

The concordance of ctDNA samples with biopsy-confirmed tumor mutations was detected in all patients with a kappa value of 0.705 (**Figure 1**), which demonstrated that plasma ctDNA could accurately mirror the profiles of the clones found in tumor tissues.

## Classification and Genotyping of the Patients With Lymphoma

According to the classification of cell origin, 6 patients were HL cases (10.2%), 17 patients were GCB cases (28.8%), 30 patients were non-GCB cases (50.8%), and 6 patients were MZL cases (10.2%). The most common subtype was DLBCL (GCB and non-GCB) (47/59, 79.7%). Distribution of lymphoma subtypes is shown in **Figure 2A**.

Genetic alterations were identified in all the ctDNA samples, and the median number of variants was 6 (range 1–16). We divided the most-affected genes of HL, DLBCL, and MZL into 14 specific pathways according to the GeneCards database. Mutations in 14 genes were identified in at least 7 patients. The six most frequently mutated genes identified in the entire group of patients (15/59–21/59, 25.4%–35.6%) were *TNFAIP3*, *MYD88*, *CD79B*, *TBL1XR1*, *TP53*, and *KMT2D*. The ctDNA mutations of different pathological subtypes in patients are shown in **Table 2**. The number of genetic mutations is shown in **Figure 2B**.

The mutated genes detected in the ctDNA of patients with HL were *SMC3* (100%), *TNFAIP3* (50.0%), and *TP53* (50.0%). Of these, *SMC3* was a mutation specific to patients with HL. The mutation of genes detected with 20% or higher ratios in patients with GCB included *CARD11* (58.8%), *MYD88* (41.2%), *TBL1XR1* (41.2%), *CD79B* (41.2%), *FAT1* (23.5%), *MALT1* (23.5%), and *ROS1* (23.5%). Mutations of *KMT2D* (56.7%), *MYD88* (46.6%), *CREBBP* (46.7%), *TP53* (36.7%), *CD79B* (36.7%), *PIM1* (30.0%), *B2M* (30.0%), *MEF2B* (26.7%), *TBL1XR1* (23.3%), *STAT6* (20.0%), *BCL6* (23.3%), *GNA13* (23.3%), *PIK3CD* (23.3%), *TNFAIP3* (20.0%), *BCL10* (20.0%), and *SYK* (20.0%) were found in 20% or more of the patients who were diagnosed with non-GCB. Mutations of *MALT1* and *ROS1* were found only in patients with GCB, and the mutations of *TET2* and *TRAF3* were present in patients with non-GCB only. Both GCB and non-GCB showed a significant difference in the mutant allele frequencies of *MALT1*, *CD79B*, *ROS1*, *TBL1XR1*, *PIM1*, *TET2*, and *TRAF3*. Mutations of *PTPN6* (100%), *TNFAIP3*, *TBL1XR1*, *SOCS1*, *CXCR4*, *CDKN2B*, *KMT2A* (all were 50.0%), and *ATM* (33.3%) were found in patients with MZL. Mutation of *TNFAIP3* was common in patients with all subtypes. The gene mutation rate and pathways of each subtype are shown in **Figure 3**. The pathway common to all patients was *NF-κB*.

All four types of lymphoma were associated with tumor inflammation promotion, but HL was mostly characterized by mutations in necroptosis, metabolism, and cell cycle occurrence, and NHL was mostly characterized by mutations in escape immune destruction, cell proliferation, and migration.

## Correlation Between Mutated Genes and Clinical Indicators

The Kaplan–Meier analysis showed that mutations in *MYD88*, *FAT1*, *MALT1*, *ROS1*, *TBL1XR1*, *CREBBP*, *KMT2D*, *TET2*, and *TRAF3* were significantly different for the progression of the patients. Their survival curves are shown in **Figure 4**.

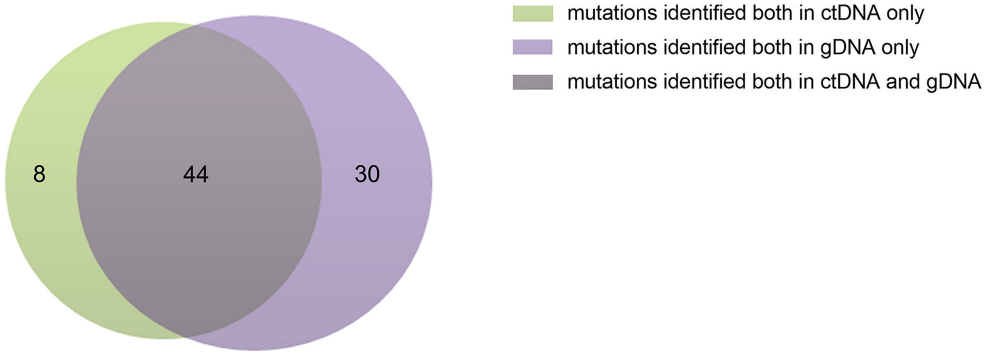


**FIGURE 1** | Numbers of ctDNA and gDNA mutations. There were 52 mutations in ctDNA and 74 mutations in gDNA, 44 mutations were common to them.

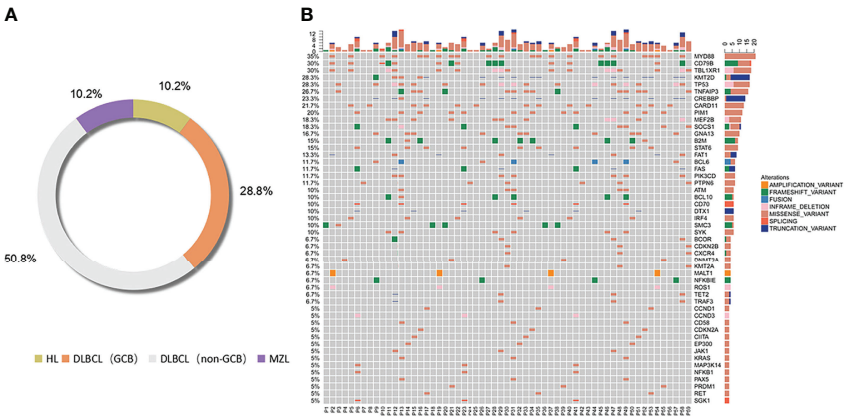


**FIGURE 2** | Distribution of lymphoma subtypes. Distribution of pathological subtypes and genetic alterations of ctDNA in the total cohort. **(A)** Detailed distribution of pathological subtypes of 59 lymphomas. **(B)** Genetic alterations of ctDNA in the total cohort. HL, Hodgkin's lymphoma; DLBCL, diffuse large B cell lymphoma; MZL, marginal zone lymphoma.

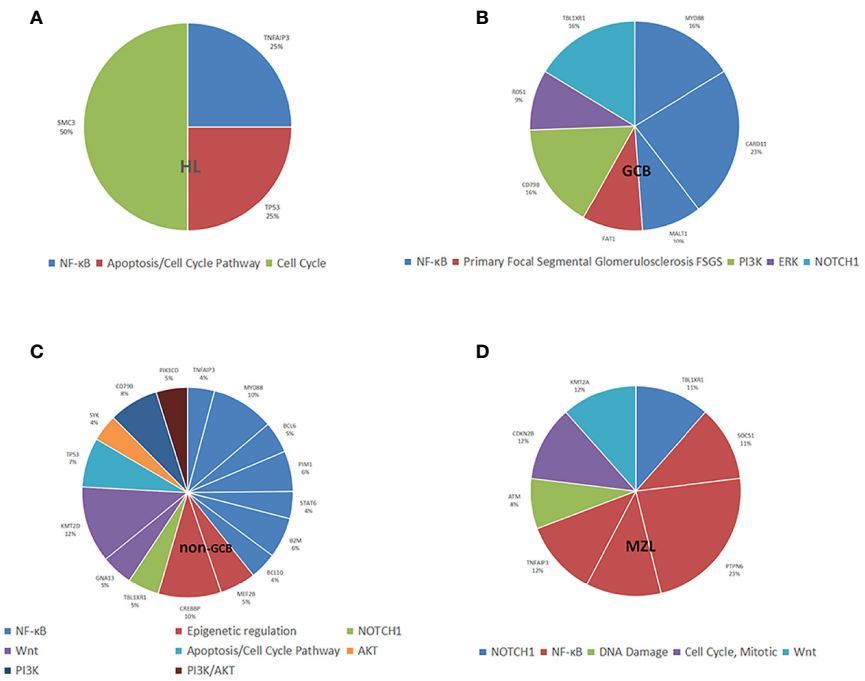


**FIGURE 3** | Distribution of mutation allele frequencies and the pathways in lymphoma patients. **(A)** Mutation allele frequencies and the pathways in HL; **(B)** Mutation allele frequencies and the pathways in GCB; **(C)** Mutation allele frequencies and the pathways in non-GCB; **(D)** Mutation allele frequencies and the pathways in MZL.

To analyze the correlations between the *MYD88*, *FAT1*, *MALT1*, *ROS1*, *CREBBP*, *KMT2D*, *TET2*, and *TRAF3* mutations and the clinical parameters of patients with lymphoma, we divided the mutations into positive and negative. **Table 3** summarizes the correlations of *MYD88*, *FAT1*, *MALT1*, *ROS1*, *CREBBP*, *KMT2D*, *TET2* and *TRAF3* mutations with the clinical parameters of patients with lymphoma, including gender, age, B symptoms, extranodal involvement, ECOG, and complications. The Chi-squared test showed that the mutation of *MYD88* had a significant correlation with ECOG score 3–4 and complications, and the mutations of *MALT1* or *ROS1* had a significant correlation with ECOG score 3–4. The mutations of *CREBBP* or *KMT2D* had a significant correlation with age >65.5 years. The mutation of *TET2* or *TRAF3* had a significant correlation with complications.

**TABLE 2** | ctDNA mutation in patients of different pathological subtypes.

| Subtype | Mutation (median) | Mutation range |
|---|---|---|
| HL | 2 | 1-3 |
| DLBCL (GCB) | 6 | 1-10 |
| DLBCL (non-GCB) | 6 | 2-16 |
| MZL | 4.5 | 1-8 |

Mutations in *TBL1XR1* are not listed since they did not correlate with clinical parameters.

## DISCUSSION

With the development in NGS technology, a comprehensive exploration of the somatic alterations within ctDNA has become increasingly accessible. The great sequencing depth used for ultra-deep sequencing makes it very powerful for profiling clinical samples, such as formalin fixed paraffin embedded and ctDNA. Greater depth of coverage also allows to pick out mutations present only in a small fraction of malignant cells. However, accurate variant calling remains challenging due to variable coverage, sequencing errors, alignment artifacts, and other issues. Lower tumor purity proportionally reduces the effective coverage of the variant alleles in tumor cells, reducing detection sensitivity (16). Bioinformatics tools mad it possible to detect VAFs of 1% or even lower. VarScan 2 performed best overall with sequencing depths of 100× and 1000× required to accurately identify variants present at 10% and 1%, respectively (17). The minimum VAF for detection of a sequence variant is not highly correlated with the percentage tumor cellularity of the specimen or the percentage of tumor cells that harbor the sequence change. In the setting of detecting minimal residual disease, accurate detection of VAFs substantially <0.01 may be required (18), with VAF sufficiently detected as low as 0.1–0.2% (19). In the study, the minimum VAF threshold was set at 0.01, thus VarScan 2 identified the variants accurately.

Our understanding of lymphoma is rapidly evolving, driven by advances in single-cell technology. Although studies have revealed some similarities between different subtypes of lymphoma, they still face challenges in terms of tumor heterogeneity. Our study performed a targeted panel sequencing of 59 patients with lymphoma on 121 key genes and analyzed their genetic alterations. Furthermore, previous studies had proved the pre-analytical stability of ctDNA under different storage conditions (20, 21), and NGS-based ctDNA analysis could reflect genetic heterogeneity among different lymphoma subtypes, indicating that ctDNA could be a noninvasive and feasible biomarker for patients with lymphoma. Analysis of ctDNA in the plasma is clinically used to identify actionable mutations, detect residual or recurrent disease and can assess the mutational heterogeneity of the entire tumor cell population. However, ctDNA analysis cannot address mutations within individual cells and cannot assess cancer phenotypes, such as the expression of drug targets and protein biomarkers. Given the heterogeneity, the fact that resistant clones of tumors may represent only a small proportion of the entire tumor and are unlikely to suffer apoptosis, the genomes of resistant tumor subclones may not be detectable at the current sensitivity limits of cell-free DNA assays. As intact cancer cells that have entered the blood, CTCs show the predictive capability of the response to drugs through analyzing protein biomarkers on CTCs and show the broad detection of mutations through genome-wide sequencing (22). CTCs are identified and sequenced to identify operable mutations in drug-resistant subclones that are not present in the majority of tumors to guide subsequent therapy. In addition, single-cell sequencing of CTC provides better access to variability between clones with different drug resistance mechanisms. Therefore, CTCs are better suited to study heterogeneity at the cellular level.

It has been reported that *MYD88* mutation was detected in the cfDNA of one patient with lymphoplasmacytic lymphoma (23). Schmitz et al. (24) studied 574 DLBCL biopsy samples using exome and transcriptome sequencing and identified four prominent genetic subtypes in DLBCL, one of which was

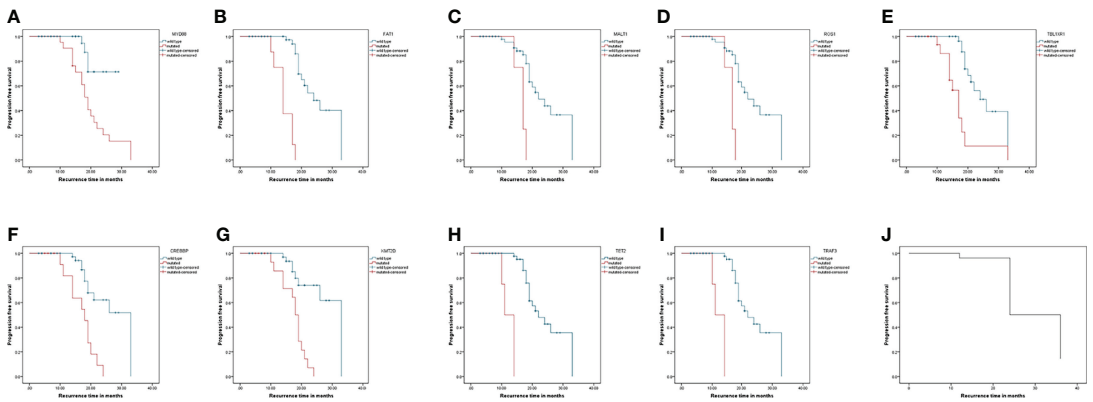


**FIGURE 4** | Progression-free survival curves of patients with lymphoma. Kaplan-Meier curves for progression-free survival by presence or absence of genes mutation. **(A–I)** was genes: MYD88, FAT1, MALT1, ROS1, TBL1XR1, CREBBP, KMT2D, TET2, and TRAF3. **(J)** was the progression-free survival curve for all patients.

**TABLE 3 |** Correlation of MYD88, FAT1, MALT1, ROS1, CREBBP, KMT2D, MALT1 and ROS1 mutations with clinical parameters of lymphoma patients.

| Clinical Parameters | n | MYD88 mutations | | $\chi^2$ | *P* | FAT1 mutations | | $\chi^2$ | *P* | MALT1 or ROS1 mutations | | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | + | - | | | + | - | | | + | - | | |
| Sex | | | | 0.167 | 0.683 | | | 0.162 | 0.687 | | | 0.075 | 0.784 |
| Male | 26 | 10 | 16 | | | 3 | 23 | | | 1 | 25 | | |
| Female | 33 | 11 | 22 | | | 5 | 28 | | | 3 | 30 | | |
| Age (years) | | | | 0.058 | 0.810 | | | 2.569 | 0.109 | | | 0.656 | 0.418 |
| >65.5 | 18 | 6 | 12 | | | 0 | 18 | | | 0 | 18 | | |
| ≤65.5 | 41 | 15 | 26 | | | 8 | 33 | | | 4 | 37 | | |
| B symptoms | | | | 2.020 | 0.155 | | | 0.604 | 0.437 | | | 0.000 | 1.000 |
| Present | 18 | 4 | 14 | | | 1 | 17 | | | 1 | 17 | | |
| Absent | 41 | 17 | 24 | | | 7 | 34 | | | 3 | 38 | | |
| Extranodal involvement | | | | 3.209 | 0.073 | | | 3.157 | 0.076 | | | 0.877 | 0.349 |
| With | 39 | 17 | 22 | | | 8 | 31 | | | 4 | 35 | | |
| Without | 20 | 4 | 16 | | | 0 | 20 | | | 0 | 20 | | |
| ECOG | | | | 4.757 | 0.029 | | | 3.131 | 0.077 | | | 11.946 | 0.001 |
| 0-2 | 47 | 13 | 34 | | | 4 | 43 | | | 0 | 47 | | |
| 3-4 | 12 | 8 | 4 | | | 4 | 8 | | | 4 | 8 | | |
| Complications | | | | 3.894 | 0.048 | | | 0.269 | 0.604 | | | 0.998 | 0.318 |
| With | 38 | 17 | 21 | | | 4 | 34 | | | 4 | 34 | | |
| Without | 21 | 4 | 17 | | | 4 | 17 | | | 0 | 21 | | |

| Clinical Parameters | n | CREBBP mutations | | $\chi^2$ | *P* | KMT2D mutations | | $\chi^2$ | *P* | TET2 or TRAF3 mutations | | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | + | - | | | + | - | | | + | - | | |
| Sex | | | | 0.011 | 0.917 | | | 0.746 | 0.388 | | | 0.000 | 1.000 |
| Male | 26 | 6 | 20 | | | 6 | 20 | | | 2 | 24 | | |
| Female | 33 | 8 | 25 | | | 11 | 22 | | | 2 | 31 | | |
| Age (years) | | | | 12.077 | 0.001 | | | 9.031 | 0.003 | | | 0.656 | 0.418 |
| >65.5 | 18 | 10 | 8 | | | 10 | 8 | | | 0 | 18 | | |
| ≤65.5 | 41 | 4 | 37 | | | 7 | 34 | | | 4 | 37 | | |
| B symptoms | | | | 0.263 | 0.608 | | | 1.863 | 0.172 | | | 0.656 | 0.418 |
| Present | 18 | 3 | 15 | | | 3 | 15 | | | 0 | 18 | | |
| Absent | 41 | 11 | 30 | | | 14 | 27 | | | 4 | 37 | | |
| Extranodal involvement | | | | 0.649 | 0.421 | | | 0.021 | 0.885 | | | 0.877 | 0.349 |
| With | 39 | 11 | 28 | | | 11 | 28 | | | 4 | 35 | | |
| Without | 20 | 3 | 17 | | | 6 | 14 | | | 0 | 20 | | |
| ECOG | | | | 0.246 | 0.620 | | | 0.001 | 0.976 | | | 0.163 | 0.687 |
| 0-2 | 47 | 10 | 37 | | | 13 | 34 | | | 4 | 43 | | |
| 3-4 | 12 | 4 | 8 | | | 4 | 8 | | | 0 | 12 | | |
| Complications | | | | 0.095 | 0.758 | | | 1.516 | 0.218 | | | 5.043 | 0.025 |
| With | 38 | 10 | 28 | | | 13 | 25 | | | 0 | 38 | | |
| Without | 21 | 4 | 17 | | | 4 | 17 | | | 4 | 17 | | |

termed "MCD" (based on the co-occurrence of *MYD88*$^{L265P}$ and *CD79B* mutations). Analysis of genetic pathways suggested that MCD relied on "chronic active" B-cell receptor signaling that is amenable to therapeutic inhibition. Our study showed similar findings that *MYD88* was the most frequently mutated gene identified in the ctDNA of patients, followed by *CD79B*, and both mutations occurred in patients with DLBCL, but not in patients with HL or MZL. This indicates that the mechanism of DLBCL development is vastly different compared with HL and MZL, and *MYD88* and *CD79B* mutations might be a major driver of DLBCL development.

Venturutti et al. (25) found through studies in mice that *TBL1XR1* alterations lead to a striking extranodal immunoblastic lymphoma phenotype that mimics the human disease. Jangam et al. (26) performed targeted deep sequencing of 8 ocular adnexal mucosa-associated lymphoid tissue lymphoma (OAML) cases, and identified *TBL1XR1* as recurrently mutated in OAML (4/8), where cases of OAML with mutations in *TBL1XR1* showed equivalent or increased vascular density compared with cases without mutations in *TBL1XR1*. Wang et al. (27) found that patients with primary testicular lymphoma with the *TBL1XR1* mutation had an inferior OS than patients with *TBL1XR1* wild type, irrespective of treatment therapy. Consistent with those studies, the present study found that patients with mutations in the *TBL1XR1* gene had significantly lower PFS rates than those without mutations, both in the population of patients with NHL and in the overall population of patients with lymphoma.

It is well known that mucosa-associated lymphoid tissue lymphoma translocator protein 1 (*MALT1*), a key adaptor protein regulating the *NF-κB* pathway, is the only protease in the pathogenesis of these related diseases. In the present study, *MALT1* mutations in the ctDNA were also found in patients with lymphoma and were only found in patients with GCB. Univariate analysis revealed that patients with *MALT1* gene

mutation had a significantly lower PFS rate than those with the wild-type *MALT1* gene (28). Therefore, *MALT1* could be a target for the treatment of GCB (29).

Nie et al. found that *CREBBP* and *EP300* genes are two frequently mutated epigenetic regulators in B-cell lymphoma and that synthesis between them is lethal (30). Mosquera et al. (31) found that mutations in *CREBBP*, *TNFRSF14*, and *KMT2D* were mainly found in follicular lymphoma, while mutations in *BTG2*, *HTA-A*, and *PIM1* were more frequent in DLBCL. In the present study, *CREBBP* and *KMT2D* appeared in patients with non-GCB, and inconsistently, *CREBBP* and *KMT2D* were mutated more frequently in patients with non-GCB than in *PIM1*. This illustrates the heterogeneity of lymphoma; there was still a high degree of heterogeneity in lymphomas of the same pathological type.

The *ROS1* fusion proteins resulting from chromosomal rearrangements of the *ROS1* gene are targetable oncogenic drivers in diverse cancers (32). Inflammatory myofibroblastic tumor fusions involving *ROS1*, *PDGFRβ*, *RET*, and *NTRK* have also been described in inflammatory myofibrosarcoma (33). Over the past few years, inhibitors of the c-Ros oncogene 1 (*ROS1*) have been approved and are currently used in clinical practice in patients with advanced non-small cell lung cancer (34, 35). However, *ROS1* mutations have not been reported in lymphoma. In the present study, as with *MALT1*, *ROS1* mutation was only found in GCB. This means that GCB has a unique *ROS1* mutation, which had a different mechanism of occurrence from other DLBCL.

Esther et al. (36)found that miR-92a and TET2 may play a synergistic role in the pathogenesis of NHL malignancies. Oreofe et al. (37)found that TET2 mutations occurred in 76% of patients with angioimmunoblastoma T-cell lymphoma (AITL). The bridging protein TNF receptor-associated factor 3 (TRAF3), as a tumor suppressor, is a key regulator of B-lymphocyte survival, and TRAF3 deficiency is sufficient to metabolically reprogram B cells (38). In this study, TET2 and TRAF3 were found to be present only in non-GCB patients, suggesting that non-GCB has a unique pathogenesis that distinguishes it from GCB and HL.

In the study, we found that all four types of lymphoma are associated with the promotion of tumour inflammation. It is well known that cancer cells, as well as surrounding stromal and inflammatory cells, are involved in carefully orchestrated interactions to form an inflammatory tumour microenvironment (TME). Cells within the TME are highly plastic, constantly changing their phenotypic and functional characteristics (39). However, each subtype has its own characteristics.

Dysregulation of apoptotic cell death mechanisms is a hallmark of cancer. Altered apoptosis is not only responsible for tumor development and progression, but also for tumor resistance to therapy. In contrast, defects in the death pathway may lead to drug resistance, thereby limiting the effectiveness of treatment (40). Therefore, a better understanding of mutations in the apoptotic pathway could improve the efficacy of cancer therapy and bypass resistance. In this study, apoptosis pathway was found in HL and non-GCB patients, which means that the population may not respond well to certain treatments and new therapeutic strategies need to be developed to counter their resistance to drugs.

In addition, the pathways in which the mutated genes are located reveal that some mutated genes in non-GCB patients are associated with epigenetic inheritance, which is completely different from the other three types. In contrast to genetic changes, epigenetic changes are reversible (41). This constitutes a promising area to understand the role of epigenetic alterations in cancer and to find new alternatives to traditional strategies (42).

Studies have shown that ECOG is an independent prognostic factor for secondary malignancies after surgery for gastrointestinal or gynecological tumors (43). ECOG is also an independent factor in the OS of patients with early onset colorectal cancer (44). In our study, we found ECOG score 3–4 was closely associated with mutations in *MYD88*, *MALT1*, and *ROS1*, which suggested that ECOG might be associated with lymphoma heterogeneity. In addition, the presence of complications was also associated with *MYD88*, *TET2*, and *TRAF3* mutations, which suggested that mutations in these genes might influence the occurrence of other complications. Furthermore, we found that mutations in the *CREBBP* and *KMT2D* genes were strongly correlated with the age of the patients, and the rate of mutations in these genes was significantly higher in patients over 65.5 years than in those under 65.5 years. Therefore, patients with co-morbidities, higher ECOG and age over 65.5 years are strongly associated with genetic mutations.

However, even though ctDNA has some advantages for patients, the potential for loss of information and the associated risks are still considered a challenge. We found a discrepancy in mutation comparisons between gDNA (FFPE samples) and ctDNA (liquid biopsies), which may lead to false-negative and false-positive results in ctDNA analysis. This is related to technical and biological factors (45). As the total number of genomic copies in the plasma volume of a sample is very limited, the number of specific variants of interest is also very limited. Also, some false negative results simply cannot be prevented, due to biological factors such as low DNA shedding in certain tumours or the location of the metastases themselves (46). In addition, multiple mutation enrichment methods and additional steps for error suppression strategies are required due to the risk of introducing errors in the library preparation or sequencing process itself (47, 48).

The stability of ctDNA varies under different conditions. Qing Kang in 2016 had processed the plasma of ten patients with metastatic breast cancer after 2, 6, and 48 h post-collection, and found that ctDNA stable for up to 6 h in both Streck and ethylenediaminetetraacetic acid (EDTA) tubes, and, one out of four patients with detectable ctDNA showed a ~ 50% decline in ctDNA in the EDTA tube after 48 h (49). Emanuela Henao Diaz found that the ctDNA levels at zero hours were not significantly different to 24- or 48-hour *in vitro* incubation in any investigated condition (50). In 2018, American Society of Clinical Oncology and College of American Pathologists jointly reviewed the information about clinical ctDNA assays and provided a

framework for future research, and indicated that testing for ctDNA was optimally performed on plasma collected in cell stabilization or EDTA tubes, with EDTA tubes processed within 6 h of collection (51). Taken together, it is a current consensus that ctDNA is stable within 6 h after the sample collection. To preserve the stability of ctDNA, we used ethylenediaminetetraacetic acid-coated tubes to collect peripheral blood samples from patients and preserved them by centrifugation within 2 hours. At the same time, an ultrasensitive method was used to detect mutations and copy number changes to ensure the stability of ctDNA in the blood stream and to reduce the errors caused by the assay.

There were also limitations to our study. In this study, the ctDNA concentration was not involved, and only the mutation abundance was detected. Since mutation abundance was not related to the ctDNA concentration, and the data were quality controlled, so the accuracy of the data could be guaranteed. In addition, the follow-up period of up to 33 months is not sufficient to demonstrate a correlation between mutations and clinical features, and a longer follow-up period is needed in future studies.

## CONCLUSION

In summary, we found that ROS1 mutations were uniquely present in GCB, while TET2 and TRAF3 were only present in non-GCB, and both MYD88 and CD79B mutations appeared only in DLBCL patients. All four types of lymphomas were associated with promotion of tumor inflammation, whereas apoptotic pathways were present only in patients with HL and non-GCB. NGS-based ctDNA mutation profiling revealed the biology of lymphoma and could identify mutational differences among lymphoma subtypes, which was a promising approach for exploring genomic evolution and discovering potential therapeutic targets, thereby facilitating personalized treatment. Future studies will require single-cell sequencing of CTCs to reveal the tole of relevant mutations in different subclones and drug resistance mechanisms.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Shanxi Bethune Hospital (YXLL-KY-2021-011). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Conception and design of the research: X-BW Acquisition of data: X-BW, NJ, WS. Analysis and interpretation of the data: X-BW. Statistical analysis: X-BW, MH. Writing of the manuscript: X-BW. Critical revision of the manuscript for intellectual content: X-BW, S-LH, Q-HZ. All authors read and approved the final draft.

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frontiers | Frontiers in Oncology

REVIEW
published: 20 July 2022
doi: 10.3389/fonc.2022.943253

# Circulating Tumor DNA as a Cancer Biomarker: An Overview of Biological Features and Factors That may Impact on ctDNA Analysis

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**Specialty section:**
This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology

**Received:** 13 May 2022
**Accepted:** 16 June 2022
**Published:** 20 July 2022

**Citation:**
Sánchez-Herrero E, Serna-Blasco R, Robado de Lope L, González-Rumayor V, Romero A and Provencio M (2022) Circulating Tumor DNA as a Cancer Biomarker: An Overview of Biological Features and Factors That may Impact on ctDNA Analysis. Front. Oncol. 12:943253. doi: 10.3389/fonc.2022.943253

Cancer cells release nucleic acids, freely or associated with other structures such as vesicles into body fluids, including blood. Among these nucleic acids, circulating tumor DNA (ctDNA) has emerged as a minimally invasive biomarker for tumor molecular profiling. However, certain biological characteristics of ctDNA are still unknown. Here, we provide an overview of the current knowledge about ctDNA biological features, including size and structure as well as the mechanisms of ctDNA shedding and clearance, and the physio-pathological factors that determine ctDNA levels. A better understanding of ctDNA biology is essential for the development of new methods that enable the analysis of ctDNA.

**Keywords: ctDNA= circulating tumor DNA, ctDNA kinetics, biomarker, liquid biopsy, monitoring**

## INTRODUCTION

Cancer ranks as the leading cause of death worldwide and the main barrier that hinders life expectancy (1). The emergence of precision medicine in the field of medical oncology brought a halo of hope for cancer patients and has improved notably in the past few decades due to the rapid expansion of knowledge in cancer genomics and the identification of targetable genomic biomarkers (2). Although the discovery of therapeutic biomarkers marked a turning point in cancer patients' treatment, several challenges arose with them. For example, in lung cancer patients, the increasing number of biomarkers to be assessed compromises the availability of tumor tissue. Moreover, tissue biopsy, apart from being a very invasive procedure that can imply potential complications for the patients, does not reflect tumor heterogeneity, making it more difficult to have an overview of the molecular characteristics of the tumor (3, 4).

In this scenario, liquid biopsy arose as a minimally invasive approach, particularly useful when tumor tissue is inadequate or non-existent, that enables the identification of significant tumor-

**Abbreviations:** bp, base pair; cfDNA, cell-free DNA; CH, clonal hematopoiesis; CRC, colorectal cancer; CTC, circulating tumor cell; CSF, cerebrospinal fluid; ctDNA, circulating tumor DNA; ddPCR, digital droplet PCR; dPCR, digital PCR; EVs, extracellular vesicles; LOD, limit of detection; MRD, minimal residual disease; mtDNA, mitochondrial DNA; NGS, next-generation sequencing; NSCLC, non-small-cell lung cancer; VAF, variant allele frequency; wt, wild-type; *ALK*, ALK receptor tyrosine kinase; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *EGFR*, epidermal growth factor receptor; *KRAS*, KRAS proto-oncogene, GTPase; *TP53*, tumor protein p53.

derived biomarkers throughout the course of the disease, including resistance mutations (5, 6). Different components can be isolated from body fluids and used in liquid biopsy analysis such as circulating tumor cells (CTCs), extracellular vesicles (EVs), tumor-educated platelets (TEPs), or circulating tumor DNA (ctDNA) (7). Among them, we are going to focus on ctDNA as it is the biomarker with more diagnostic and prognostic potential.

The cell free-DNA (cfDNA) was first described in healthy individuals by Mandel et al. in 1948 (8) and it was not until the year 1977 that Leon et al. found out increased levels of cfDNA in the serum of cancer patients (9), highlighting its huge potential as a tumor biomarker. cfDNA is generally at a concentration between 0 and 100 ng/mL in the blood of healthy patients, and is upped to >1000 ng/mL in cancer patients (4, 10). The fraction of plasma cfDNA derived from tumor cells, known as ctDNA, is the most extensively studied and the most used non-invasive alternative, from a clinical point of view, for the molecular characterization of solid tumors, including non-small-cell lung cancer (NSCLC), colorectal cancer (CRC), breast cancer (11), head and neck (12) and melanoma (13). ctDNA was first validated in clinical oncology by examining the *KRAS/BRAF* mutation in CRC patients (14) and then, it was introduced into clinical practice for the detection of mutations in the *EGFR* gene in NSCLC (15). Since then, the interest in this biomarker has exponentially risen being the topic of more than 870 publications in 2021 (Web of Science™ database, **Figure 1**) and being currently used in 359 different trials, listed in the database ClinicalTrials.gov.

Although liquid biopsies often refer to blood biopsies, other biofluids such as urine, saliva, cerebrospinal fluid (CSF), pleural effusion, pericardial effusion, and ascites effusion, can be also used (16, 17). In this way, malignant effusions that occur as a consequence of disease progression are highly informative. Indeed, tumors shed higher amounts of ctDNA into nearby body fluids than into the bloodstream (18). Moreover, peritoneal washings, which are routinely performed in surgeries of ovarian cancer patients, have been shown to be useful for BRCA testing (19). Therefore, although obtaining these biofluids may be a more aggressive procedure, they constitute an informative source for biomarker testing (18).

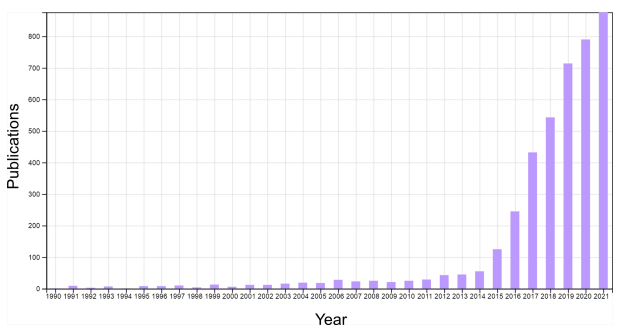


**FIGURE 1** | ctDNA publications: Number of publications with ctDNA as a topic between 1990 and 2021, collected in Web of ScienceTM database.

The study of ctDNA has multiple potential uses in oncology such as early diagnosis, tumor molecular profiling, or early detection of resistance mutations. ctDNA levels correlate well with tumor bulk and therefore it can be used as a surrogate for tumor size and staging (20, 21). In the same way, fluctuations in ctDNA levels have been shown to correlate well with the course of the disease, being an adequate approach for noninvasive tumor response to treatment monitoring for many cancer types (22–24).

However, the structure and origin of ctDNA, as well as the mechanisms of ctDNA shedding, filtering, degradation, and clearance remain unclear. In this review, we summarize the dynamics of extracellular tumor DNA, including the balance between ctDNA release and clearance and the influence of clinicopathological factors in these processes.

## ctDNA: CHARACTERISTICS AND MECHANISMS OF RELEASE

cfDNA comprises small fragments of double-stranded nuclear (coding and non-coding) and mitochondrial DNA (mtDNA) of approximately 40-200 base pairs (bp) in size, with a peak at about 166 bp that corresponds with nucleosome-associated DNA fragments (4, 25). Although the main source of cfDNA is the hematopoietic system (55% white blood cells and 30% erythrocyte progenitors) (25), there is still a huge interest in understanding how different organs contribute to the overall amount of cfDNA in the physiological and pathological conditions.

ctDNA can be released by a multitude of mechanisms, not only when cells die *via* apoptosis, necrosis, oncosis, ferroptosis, pyroptosis, and phagocytosis, but also by senescence or the active secretion in extracellular vesicles (EVs) and mtDNA egestion (26, 27) (**Figure 2**).

Fragment length and nucleosome occupancy might provide clues for cfDNA shedding mechanisms. Indeed, short fragments of <200 bp are assumed to be released during apoptosis as a consequence of caspase-dependent cleavage. Multiple of these fragments are packed in apoptotic blebs and phagocytized by macrophages, to be finally released into the blood and lymphatic circulation (28, 29). Interestingly, shorter fragments (<100 bp) might be enriched with ctDNA and mtDNA which preferentially carried tumor-derived genomic alterations (30, 31). Indeed, a higher ctDNA fragmentation pattern was observed in melanoma, lung cancer, and metastatic CRC patients with high levels of mutation burden compared with healthy individuals (32). Conversely, large fragments of >200 bp are originated during the necrosis process (33), however, the contribution of necrosis in the amount of cfDNA remains unclear (34). Interestingly, DNA of necrotic cells can be further degraded by DNase I, and necrotic cells can be engulfed by macrophages, originating smaller fragments of circulating DNA (35, 36). For all those reasons, the development of methods based on cfDNA size and fragmentation pattern is crucial to enhance the enrichment of ctDNA and

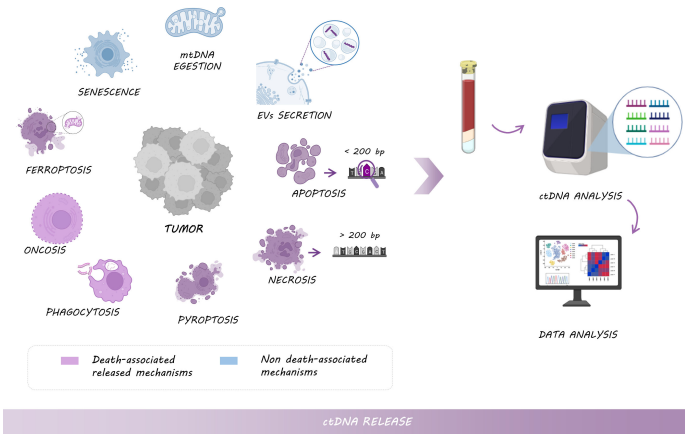


**FIGURE 2** | Mechanisms of ctDNA release: Tumor cells shed DNA into the bloodstream by different types of cell death, including necrosis (larger fragments of >200 bp), apoptosis (shorter fragments of <200 bp enriched with tumor-derived genomic alterations), pyroptosis, phagocytosis, oncosis or ferroptosis, but also by non-death associated mechanisms such as senescence, or the active secretion of EVs and mtDNA. Thus, ctDNA analysis provides tumor-relevant clinical information.

consequently, improve the sensitivity of methods for ctDNA analysis. Furthermore, the distribution of cfDNA fragments with different sizes is important, since it reflects cfDNA integrity. Although ctDNA derived from apoptotic bodies would be more informative in terms of tumor molecular information, cfDNA integrity seems to be higher in cancer patients compared to healthy individuals, suggesting that necrotic cell death plays an important role in ctDNA release, especially in advanced stages and aggressive tumors (37, 38). This observation could be explained by the fact that healthy cells die primarily by apoptosis, while malignant cells die not only from apoptosis but also from necrosis or autophagy (9). In this regard, cancer cells could activate autophagy to obtain an alternative energy source from the digestion of their damaged organelles or their self-digestion, shedding ctDNA as a consequence (39).

DNA from necrotic or apoptotic cells can be also released into the circulation by different immune cell types; however, it is not clear how much each mechanism contributes to the amount of ctDNA. After the phagocytosis of necrotic and apoptotic cells, macrophages or other scavenger cells digest the DNA into smaller fragment sizes and release them into the tissue microenvironment and bloodstream, actively or dying (36, 40).

ctDNA can also be actively released by living tumor cells, from primary tumors or metastases *via* EVs. Like cfDNA, there is a wide variety of EVs in terms of size whose role in cancer and, specifically, in the transport of ctDNA between distant tissues for cell communication seems to differ. In this sense, Vagner et al. showed that both ctDNA and EVs size seems to be a key element in genomic alteration transport (41). Indeed, large vesicles (from 100 nm up to 1 μm in diameter) from prostate cancer patients, such as microvesicles or apoptotic bodies, appear to be enriched with smaller fragments of ctDNA (<200 pb), compared with small EVs, from 30 to 150 nm in diameter, such as exosomes (41, 42). Still, nanoscale EV-derived DNA (approximately 114 nm average size in stage-I EV samples) has been demonstrated to be a superior mutation detection method in early-stage NSCLC compared to cfDNA (43). In line with these data, additional studies have identified the presence of DNA in EVs isolated from cancer patient samples and described the identification of different mutations in oncogenes such as *KRAS* or *TP53* (44–47). However, the proportion of ctDNA engulfed into EVs actively released by tumor cells and the effect of different treatments on this active secretion is not clear (41, 48).

Irrespective of the mechanism of ctDNA shedding; nucleosome footprints, DNA methylation profiles, DNA preferred end motifs, and genetic alterations can be used to characterize and identify the origin of cfDNA as they carry information from the original tissues (49–51) (**Figure 3**). In this regard, certain human genomic locations have been described as preferential ends when ctDNA is generated (52), suggesting that DNA cleavage is a non-random process. Interestingly, a greater end motif diversity has been associated with cancer patients (53, 54), suggesting that ctDNA tail motifs could be used to enhance the performance of cancer diagnosis by identifying the fragments of cfDNA from tumor cells (ctDNA) and filtering out fragments from healthy cells. Another interesting approach for determining the tissue source of ctDNA was proposed by Snyder et al. (49), who hypothesized that it is feasible to identify cfDNA origin based on nucleosome positioning. Nucleosomes are distributed along DNA following different patterns that correlate with characteristic epigenetic features of different cell types or even according to cancer types. Matching the epigenetic footprint of these ctDNA fragments against reference databases would enable the molecular classification of cancers of unknown origin. In line with these data, the stability of DNA methylation and the presence of cell-specific methylation patterns can also contribute to the identification of tumor origin or even the detection of metastasis through cfDNA analysis (55). Specifically, the analysis of differentially methylated regions in colon and liver tissues enabled the differentiation of patients with

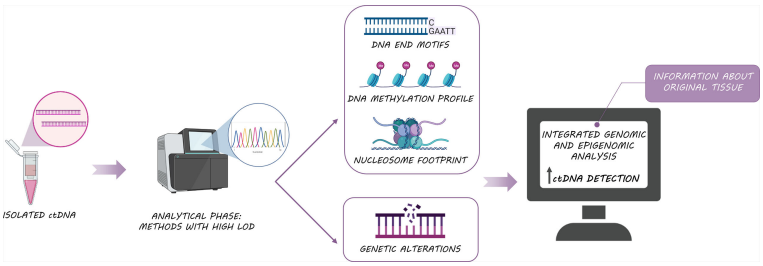


**FIGURE 3** | Biological features of ctDNA: The integrated analysis of ctDNA somatic alterations, methylation and fragmentomic information, improves ctDNA detection and provides useful information about original tissue.

liver or colon cancer but also, the discrimination between colon cancer patients with and without liver metastasis (56). Indeed, the analysis of cfDNA methylation has been already approved by the FDA for its use in the clinic (57), being the Epi proColon test the first screening analyzing a cfDNA methylation biomarker approved in 2016 for colorectal cancer patients (58).

## ctDNA CLEARANCE

The amount of cfDNA, and ctDNA in particular, depends on a balance between DNA shedding and DNA clearance. Overall, the half-life of cfDNA ranges from 16 minutes to 2.5 hours (59), as a consequence of the action of three main different mechanisms: (i) the action of DNases present in the bloodstream (60), (ii) the active clearance of nucleosomes and DNA and (iii) filtration in organs such as kidney or lymph nodes (**Figure 4**).

ctDNA clearance can be carried out by various filtering organs (60). Kupffer cells within the liver are responsible for clearing the majority of cfDNA, specifically longer fragments (61, 62), followed by kidneys, which are involved in DNA fragmentation through their deoxyribonuclease activity (62). In this way, *in vivo* experiments injecting radiolabeled mononucleosomes in mice demonstrated that the liver removed approximately 70 to 85% of the nucleosomes within 10 min (61). The macrophages of the spleen and lymph nodes play also a minor role in ctDNA clearance (**Figure 4**). In addition to these organs, lymphatic drainage may constitute the main source of ctDNA clearance within the tumor microenvironment (60). In cancer patients, cfDNA level is higher than in healthy individuals in part due to the excess of cell death by the whole set of mechanisms aforementioned, which leads to the overload of the clearance systems and subsequent accumulation. Nevertheless, the kinetic dynamics of ctDNA in cancer patients need to be further studied.

Finally, the association with molecular or macromolecular complexes, as well as encapsulation in EVs, prevent the rapid degradation of ctDNA by circulating enzymes and immune system cells (63). Another factor that seems to play a role in ctDNA clearance is fragment size, but it is still not clear how they affect half-life.

## ctDNA LEVELS IN DIFFERENT TYPES OF CANCER

The first time that cfDNA was measured in different cancer types was in 1977 by Leon et al. (9), who reported that levels of cfDNA in patients with various cancers were higher compared with

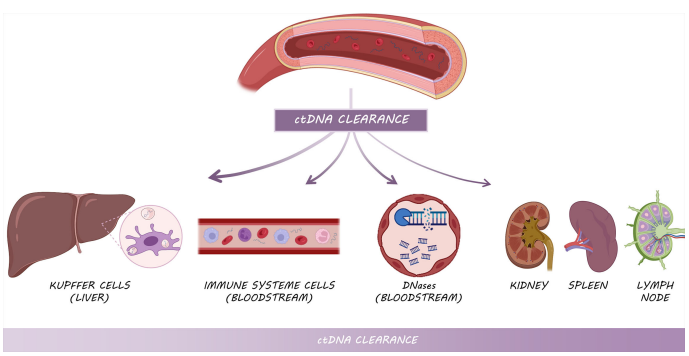


**FIGURE 4** | Mechanisms of ctDNA clearance: Kupffer cells from the liver are primarily responsible for ctDNA clearance, followed by circulating enzymes and immune system cells and other filtering organs such as kidneys, spleen and lymph nodes.

healthy individuals. A few years later, in 1989, Stroun et al. (64) stated that increased levels of cfDNA in cancer patients were caused by a fraction of DNA released into the bloodstream by cancer cells, this portion of cfDNA was named ctDNA. Nowadays, it is well established that ctDNA levels vary depending on the cancer type (**Figure 5**). It has been especially characterized that tumors located in the central nervous system release the lowest levels of ctDNA into the bloodstream due to the blood-brain barrier (21, 65, 66). Of note, more than 90% of patients with gliomas did not harbor detectable levels of ctDNA according to Huang et al. (67). Similarly, Zill et al. (68) analyzed 25,578 samples from 21,807 patients in more than 50 tumor types, reporting a ctDNA detection rate of 93%. Remarkably, no differences were found in terms of ctDNA detection except for patients with brain tumors or brain-only metastases, who shed significantly less ctDNA into the bloodstream. Likewise, some studies have also pointed out that patients with visceral metastases have higher levels of ctDNA than those with brain metastases (69–72). Noteworthy, about 30% of cancer patients develop intracranial metastases, a severe complication that decisively affects the patient's prognosis and quality of life (73,74). Thus, it would be important to optimize the detection of ctDNA for these patients in other body fluids such as CSF. In addition, ctDNA detection is rather challenging in medulloblastomas, or kidney, prostate or thyroid cancer. On the other hand, ctDNA can be easily detected in samples from advanced stages of ovarian, liver, pancreas, bladder, colon, lung, stomach, breast, liver, esophagus, and head and neck cancer patients as well as neuroblastoma and melanoma patients (21).

Interestingly, there are also variations within the same type of cancer depending on tumor histology. For example, in lung cancer, a higher percentage of ctDNA detection has been described in squamous tumors compared to adenocarcinomas. The most plausible explanation is that squamous tumors have a more necrotic profile (75). These results have also been observed in patients with triple-negative breast cancer, whose ctDNA levels are higher than those of other breast cancer subtypes, which can be related to a higher rate of necrosis and cell proliferation (76, 77).

Lastly, tumors harboring certain alterations such as *TP53* mutations and copy number gains seem to have increased ctDNA shedding, which may be due to increased metabolic activity or cellular turnover (71). In this regard, *TP53* alterations have been suggested to be markers of aggressiveness and poor prognosis (78).

## ctDNA AS A SURROGATE OF TUMOR BURDEN, STAGE, AND METASTASIS

The amount of ctDNA has been associated with tumor size, stage, and metastasis in multiple studies (**Figure 5**). Specifically, a retrospective study of serially collected liquid biopsy samples from 40 ovarian cancer patients demonstrated a significant correlation between lesion volume and ctDNA level (79). Another study that analyzed samples from 640 patients with different tumor types described a clear correlation between ctDNA and cancer stage, reaching higher levels in patients with advanced disease and lower levels in premalignant and early-stage cancers (21). In line with these results, analyzing samples from more than 20,000 patients with different tumors, Zill and colleagues showed that those patients with premalignant lesions or earlier stages shed less ctDNA than those with advanced stages (68). Specifically, in NSCLC patients, Chabon et al. were able to detect ctDNA in 42%, 67%, and 88% of patients with stage I, II, and III diseases respectively (80). In fact, 50% of localized tumors shed ctDNA without reaching 0.01% of the

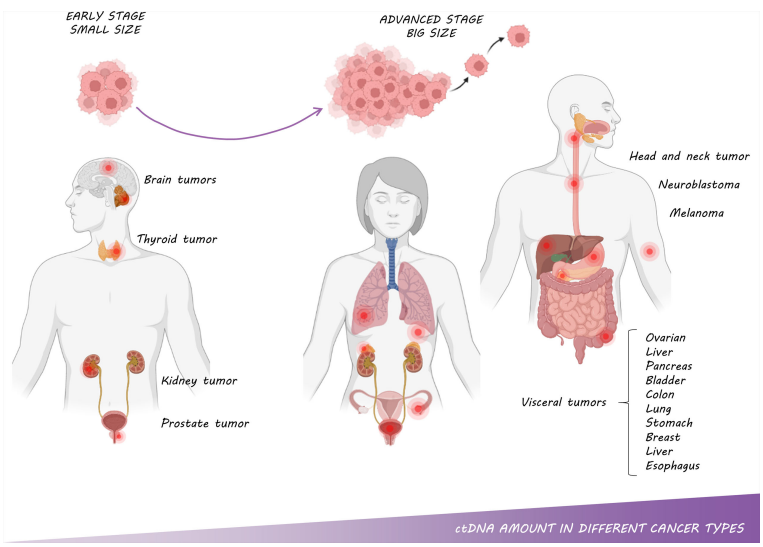


**FIGURE 5** | ctDNA amount in different cancer types: The amount of ctDNA is correlated with tumor type, size, stage and metastasis.

ctDNA level (80); whereas advanced-stage tumors release concentrations of ctDNA than can exceed 10% of the cfDNA (21).

On the other hand, in ctDNA-positive patients, tumor size and volume correlate broadly with ctDNA levels, as measured by the mean of variant allele frequency (VAF) of single nucleotide variants detected in plasma ctDNA (81). Currently, it is not well established how ctDNA should be quantified. This issue is especially controversial in tumors that do not harbor druggable mutations. In this sense, it is not clear whether it is more appropriate to select the highest VAF among all detected mutations or to take all of VAFs into account through summation, arithmetic mean, or other approaches.

In summary, ctDNA levels increase proportionally according to tumor burden, disease stage, and metastasis, highlighting the use of ctDNA as a prognostic biomarker. Indeed, it is well established that patients with high levels of ctDNA have worsened survival outcomes compared with those with lower or even undetectable levels of ctDNA (82–85).

## ctDNA TO MONITOR TREATMENT OUTCOMES

Numerous studies show that ctDNA levels correlate well with tumor load and therefore ctDNA dynamics can be used as a surrogate of treatment response (86–88). In addition, the modification of the ctDNA methylation profile has been proposed as an alternative biomarker for treatment response (89). It has been shown that the type of treatment, as well as the time interval between exposures and the dose, may rate affect ctDNA shedding. In this way, it has been suggested that targeted therapies used in cancer patients, such as *EGFR* or *ALK* tyrosine kinase inhibitors, promote faster ctDNA clearance than immunotherapy (90). Furthermore, cytotoxic therapies such as chemotherapy or ionizing radiation seem to increase cfDNA levels due to cellular senescence (91, 92). Of note, it is well established that some chemotherapy agents produce leukopenia. cfDNA from dying cells dilutes ctDNA in wild-type (wt) DNA leading to decreased levels in VAF, which may bias results. Conversely, other cancer treatments do not release as much cfDNA due to its mainly cytostatic effect, implying cell growth arrest (93). In the neoadjuvant setting, ctDNA has been shown to correlate well with tumor response to treatment. Recently, NADIM investigators have shown that ctDNA clearance after neoadjuvant chemo-immunotherapy outperformed tumor response to treatment measured by CT-scans and according to RECIST criteria in the prediction of survival (94). Similarly, a significant association between pathological complete response and ctDNA clearance was reported in the CheckMate 816 trial (95). Measurement of residual disease following neoadjuvant treatment that accurately predicts long-term survival is an essential requirement for clinical trial development. Although further studies are needed, ctDNA postulates as an early surrogate of survival being a promising trial endpoint in the neoadjuvant setting.

Finally, patients with surgically resected tumors show a sharp drop in ctDNA levels after surgery (59). However, the amount of nonspecific cfDNA increased after tumor resection (96), due to injury of surrounding tissue during surgery. In this sense, the appropriate time point for plasma collection after surgery needs to be established. In these patients, ctDNA detection allows monitoring of minimal residual disease (MRD) after tumor resection (97). Several platforms with exceptional sensitivities such as cancer personalized profiling by deep sequencing (CAPP-Seq) (98) targeted error correction sequencing (TEC-Seq) (99), the Tracking Cancer Evolution Through Therapy (TRACERx, Signatera) (81, 100) or CancerSEEK5 multiplex PCR (mPCR) (101) have been shown to be useful for detection of minimal residual disease (MRD) or early detection of cancer. In this regard, it appears that measuring not just ctDNA can boost sensitivity. Combining ctDNA analysis with the study of informative methylation regions improves sensitivity (102) (**Figure 3**).

## TECHNICAL FACTORS AFFECTING ctDNA DETECTION

The use of ctDNA to noninvasively assess tumor genomic variants is increasing. However, some pre-analytical and analytical issues may affect the detection and quantification of ctDNA.

Regarding starting material, blood plasma is a preferential choice compared with serum because wt cfDNA released from leukocytes during the clotting process in serum samples dilutes ctDNA in wt DNA (103, 104). Particularly, Soo et al. reported a higher level of cfDNA in serum (481 ng/mL) than in plasma (17.7 ng/mL). Of note in a cohort of 33 pre-treatment serum and 75 pre-treatment plasma samples from patients with diffuse large B cell lymphoma, Soo et al. were able to detect more genomic alterations in plasma samples (186 *vs.* 22 mutations) with higher tumor allele fraction (2.8% *vs.* 0.85%) (105), compared with serum samples. In addition, plasma samples have shown less inter-patient variability (106). The use of $K_2$ EDTA tubes to collect plasma samples is therefore recommended when samples are processed within 6 hours after blood extraction (107). For longer periods between extraction and processing, the use of special collection tubes with stabilizing agents is recommended. Of note successful preservation of cfDNA over 14 days at room temperature is possible using collection tubes with stabilizing agents (108).

Concerning sample processing, the complete removal of any cellular component is essential. For this goal, the best option is a two-step centrifugation at 1600g for 10 minutes for plasma isolation (109). According to this recommendation, Herrera et al. reported less concentration of cfDNA in plasma samples that were centrifuged twice compared with samples that were centrifuged only once (13 μg/l *vs.* 819 μg/l), revealing that cfDNA concentrations were contaminated with genomic DNA (110). These observations confirm that the second centrifugation step is crucial for ctDNA analysis. Finally, it is well known that ctDNA

integrity is better conserved as cfDNA extracts compared to plasma when samples are stored at -80°C and avoiding freeze-thaw cycles (103).

As already mentioned, body fluids other than blood have shown a higher concentration of cfDNA compared to blood samples in patients with lung adenocarcinoma with *EGFR* mutations (1.90 vs. 0.36 ng/μL; p=0.0130). Likewise, CSF from patients with primary brain tumors such as glioblastoma, glioma, or primary central nervous system lymphoma showed higher amounts of ctDNA compared to peripheral blood (18, 19).

Technical procedures for cfDNA isolation can be classified into three categories: phase isolation, silicon membrane-based spin column, and magnetic bead-based isolation. Phase isolation methods may lead to a high cfDNA isolation yield and a wide range of DNA fragment sizes. On the other hand, spin column and magnetic bead-based methods have lower efficiency but show a higher selective recovery for DNA fragments of a certain size (111, 112). Specifically, cfDNA purification using magnetic beads appears to recover higher amounts of small cfDNA fragments compared to silica membrane methods (113). In any case, automated processing should be performed to reduce operator variability (114). Nevertheless, within automatic methods, Pérez-Barrios et al. reported different recovery of mono-, di- and tri-nucleosomes DNA fragments when analyzing 34 cfDNA samples obtained from 17 plasma samples from cancer patients extracted by Maxwell® RSC ccfDNA Plasma Kit (Promega Corporation, Madison, WI, USA) and MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Penzberg, Germany) methodologies (112).

Currently, several platforms are available for noninvasive biomarker testing some of which have received approval from regulatory agencies. There is a wide range of reported sensitivities of the different methodological approaches, in this way PCR-based approaches have a significantly lower limit of detection (LOD) compared to other technologies such as dPCR and NGS (115). Although dPCR offers an ultra-high sensitivity for ctDNA analysis, only a few known mutations can be tested at a time, whereas NGS technologies allow the screening of multiple genomic alterations, known or unknown. In addition, NGS enables the combination of genomic data and epigenomic signatures, which may improve sensitivity (116) (**Figure 3**). In any case, the knowledge of the limitations of the different technical approaches for ctDNA analysis is crucial for the accurate interpretation of the results (117).

cfDNA input remains the major limiting factor, and for most techniques using less than 20 ng of cfDNA may impair results. A study by Zhang Y et al. showed that the sensitivity declined from 82.6% to 46.7% when using cfDNA inputs of ≥ 5 ng per reaction and < 2 ng, respectively (118). Furthermore, several comparative studies have clearly reported that, among other technical factors, discordant calls mostly occur at low VAF (115, 119), and therefore VAFs should always be reported in clinical reports.

Finally, it is important to point out that clonal hematopoiesis (CH) constitutes an important source of false-positive calls. CH is defined by the presence of a somatic mutation in blood or hematopoietic progenitor cells, but without other diagnostic criteria for hematological malignancy. It is more frequent in aged patients and patients with solid tumors and of course, it is more likely to be detected with deeper sequencing approaches (120). Importantly, CH-derived mutations can lead to erroneous sequencing results which thereby might guide erratic treatment recommendations (121).

## CONCLUSIONS

Liquid biopsy overcomes some tissue biopsy limitations such as tumor heterogeneity, tissue availability, and risks associated with the invasive procedure. Among the biological components of body fluids, ctDNA has emerged as a pivotal analyte for the management of cancer patients. However, ctDNA detection and quantification are affected by several physio-pathological conditions and a deeper knowledge of factors affecting ctDNA kinetics is needed. The size fragment pattern, nucleosome, and methylation profile of ctDNA may differ according to the original tissue and the mechanism of release, which may be clinically informative, and methodological approaches capable to explode this information are of particular interest.

## AUTHOR CONTRIBUTIONS

ES-H, RS-B and AR drafted and critically revised the manuscript. All authors reviewed the draft. All authors contributed to the article and approved the submitted version.

## FUNDING

ES-H was funded by the Consejería de Ciencia, Universidades e Innovación of the Comunidad de Madrid (Doctorados Industriales of the Comunidad de Madrid IND2019/BMD-17258). RS-B was funded by the Ministerio de Ciencia e Innovación (Programa Estatal de Investigación, Desarrollo e Innovacion Orientada a los Retos de la Sociedad, RTC2019-007359-1).

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**Conflict of Interest:** Authors ES-H and VG-R were employed by Atrys Health. AR reports the following conflict of interest: Consulting or Advisory Role: Takeda, AstraZeneca. Research Funding: Bristol Myers Squibb Foundation (Inst), Boehringer Ingelheim (Inst), Takeda (Inst) Expert Testimony: Vivo Diagnostics. MP reports grants, personal fees, and travel expenses from BristolMyers Squibb, Roche, and AstraZeneca; and personal fees from Merck Sharpe & Dohme and Takeda, outside the submitted work.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Oncology

TYPE Review
PUBLISHED 29 September 2022
DOI 10.3389/fonc.2022.970242



OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cancer Molecular Targets and Therapeutics, a section of the journal Frontiers in Oncology

RECEIVED 15 June 2022
ACCEPTED 15 September 2022
PUBLISHED 29 September 2022

CITATION
Lam RCT, Johnson D, Lam G, Li MLY, Wong JWL, Lam WKJ, Chan KCA and Ma B (2022) Clinical applications of circulating tumor-derived DNA in the management of gastrointestinal cancers – current evidence and future directions.
*Front. Oncol.* 12:970242.
doi: 10.3389/fonc.2022.970242



# Clinical applications of circulating tumor-derived DNA in the management of gastrointestinal cancers – current evidence and future directions

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Advances in Next Generation Sequencing (NGS) technologies have enabled the accurate detection and quantification of circulating tumor-derived (ct)DNA in most gastrointestinal (GI) cancers. The prognostic and predictive utility of ctDNA in patiets with different stages of colorectal (CRC), gastro-esophageal (GEC) and pancreaticobiliary cancers (PBC) are currently under active investigation. The most mature clinical data to date are derived from studies in the prognostic utility of personalized ctDNA-based NGS assays in the detection of minimal residual disease (MRD) and early recurrence after surgery in CRC and other GI cancers. These findings are being validated in several prospective studies which are designed to test if ctDNA could outperform conventional approaches in guiding adjuvant chemotherapy, and in post-operative surveillance in some GI cancers. Several adaptive studies using ctDNA as a screening platform are also being used to identify patients with actionable genomic alterations for clinical trials of targeted therapies. In the palliative setting, ctDNA monitoring during treatment has shown promise in the detection and tracking of clonal variants associated with acquired resistance to targeted therapies and immune-checkpoint inhibitors (ICI). Moreover, ctDNA may help to guide the therapeutic re-challenge of targeted therapies in patients who have prior exposure to such treatment. This review will examine the most updated research findings on ctDNA as a biomarker in CRC, GEC and PBCs. It aims to provide insights into how the unique strengths of this biomarker could be optimally leveraged in improving the management of these GI cancers.

KEYWORDS

**ctDNA, gastrointestinal cancer, minimal residual disease, prognostic and predictive biomarker, next generation sequencing**

# 1 Introduction

According to GLOBOCAN 2020, several gastrointestinal (GI) cancers are amongst the top ten most prevalent and lethal cancers in certain parts of the world (1). Colorectal cancer (CRC) accounts for one in every 10 cancer-related deaths and is most prevalent in Western countries. Gastric cancer (GC) and esophageal squamous cancer (ESCC) are more common in East Asia and are responsible for one in every 13 and one in 18 cancer-related deaths in the world, respectively. The overall incidence rates of CRC, pancreatic cancer (PC) and biliary cancers (BLC) are stable or declining, but GC and esophageal adenocarcinoma (GEA) show rising trends in younger people from developed countries (1). Systemic therapy is integral to the management of some advanced GI cancers and the use of biomarkers in guiding treatment decisions may improve patient's outcome (2–4).

Circulating tumor DNA (ctDNA) is a non-invasive and promising biomarker which is under active investigation in patients with GI cancers. The term 'liquid biopsy' refers to the process of sampling ctDNA, which is a component found in cell-free DNA (cfDNA) originating from the direct release, active secretion, necrosis or apoptosis of tumor cells into the circulation (5, 6). Each fragment of ctDNA usually has an average size of 166 base pairs, which resembles mononucleosomal units originating from cellular apoptosis (7). In recent years, research studies have evaluated the utility of ctDNA in the management of some GI cancers in these clinical settings: 1) the detection of minimal (or molecular) residual disease (MRD) following surgical resection of the primary tumor and in guiding adjuvant therapy; 2) assessment of clinical response to neoadjuvant chemotherapy and/or radiotherapy; 3) monitoring of response to palliative drug therapies; 4) tracking of clonal dynamics and evolution during targeted therapy, as well as in 5) the enrichment and selection of patients for clinical trials of novel anti-cancer therapies (Figure 1). The main objective of this article is to review the latest and most salient research studies on the clinical application of ctDNA in patients with advanced CRC, GEA, ESCC, PC and BLC. This review will also focus on how the strengths of ctDNA can be optimally leveraged in improving the treatment of these GI cancers.

# 2 Overview of ctDNA as a biomarker in gastrointestinal cancers

The quantification of ctDNA in solid tumors generally involves two broad categories of assays: tumor-informed and tumor-agnostic assays (8, 9). Tumor-informed assays require a prior knowledge of tumor-specific genomic alterations. One of the commonest platforms used is the polymerase chain reaction (PCR)-based assays, which include droplet digital PCR (ddPCR), quantitative real-time (RT-qPCR) and 'Beads, Emulsion, Amplification and Magnetics' (BEAMing) PCR (8, 9). Another type of platform is to apply Next-Generation Sequencing (NGS) on a target panel of genomic alterations, examples of which include the Tagged-Amplicon deep sequencing (TAm-seq), Safe-sequencing System (Safe-SeqS) and CAncer Personalized Profiling by deep sequencing (CAPP-Seq) (8, 10). Such NGS-based assays are highly sensitive with a Limit of Detection (LOD) of variant allelic frequencies (VAF) as low as 0.01%, and specific in detecting various mutations including indels, rearrangements and copy number alterations (CNAs) in GI cancers. In contrast, tumor-agnostic assays are broad, panel-based sequencing assays that detect genomic alterations and methylation changes (9).

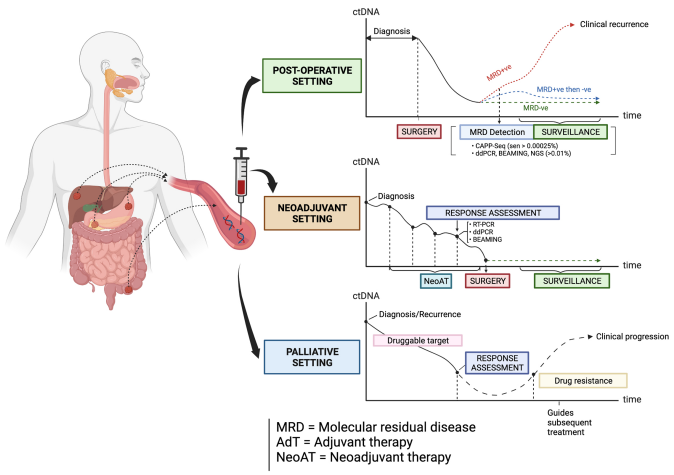


FIGURE 1
The types of clinical settings where ctDNA is being investigated as a biomarker in guiding the management of some gastrointestinal cancers. Created with BioRender.com.

They allow real-time tracking of novel mutational changes and cancer-specific variants simultaneously (8, 9).

In general, the detection rates of ctDNA can vary between different types of GI cancers. Bettegowda et al. found that ctDNA could be detected in around 73%, 57% and 48% of patients with CRC, GEC and PC, respectively (11). Strickler et al. reported a high correlation between the rates of ctDNA-derived and tumor-derived NGS-based detection of 20 most commonly mutated genes in CRC (12). However, the detection and interpretation of ctDNA are potentially limited by several patient-related and assay-related factors. Discordance between tumor and plasma samples may be influenced by intra-tumoral heterogeneity, tumor histology, anatomical location of metastases and the patient's tumor burden. For instance, GC has a higher level of genomic heterogeneity than PC and CRCs, resulting in more variable interpatient rates of ctDNA detection. The level of tumor DNA shedding into plasma is lower with mucinous tumors and locoregional metastases, compared with liver metastases in CRC (11, 13, 14).

Limitations resulting from these pre-analytical and assay-related factors may undermine the accuracy of ctDNA results. False-negative results may be caused by the low VAF of specific variants or from inadequate volumes of plasma sampled (9). Since cfDNA is also released by blood cells, the expansion of blood cells in clonal hematopoiesis of indeterminate potential (CHIP) may increase the level of background noise signals and false-positive ctDNA measurements (15). According to a consensus statement by the National Cancer Institute (NCI) Colon-Rectal-Anal Taskforce, limitations related to ctDNA assays could be minimized by standardization of a common protocol for blood collection, sample processing, DNA extraction and analysis (16).

# 3 Colorectal cancer

To date, the most mature clinical data on ctDNA are derived from patients with CRC. The detection rate of ctDNA in CRC is relatively high compared with other GI cancers that are discussed in this review - from an overall 73% in localized CRC (10), to 95.8% in patients with liver metastasis (16). Figure 2 is a chronological overview of some of the key studies on the clinical application of ctDNA in the management of early and advanced CRC. Details of these studies will be discussed in the following sections.

## 3.1 Detection of minimal residual disease after surgery to guide adjuvant chemotherapy and surveillance for recurrence in early colorectal cancer

ctDNA allows the detection of MRD - residual cancer cells that are not detectable by conventional diagnostic tools (17). The current standard of care for stage III and some high-risk stage II colon cancers are surgery followed by adjuvant chemotherapy and then surveillance. The NCI Colon-Rectal-Anal Taskforce recommends the minimum time-points for perioperative sample collection to be 4-8 weeks post-resection, as cell damage during surgical resection and wound healing may lead to a surge in cfDNA (16).

### 3.1.1 Earlier studies on prognostic significance of minimal residual disease after surgery

Earlier trials have consistently shown that ctDNA is a powerful prognostic biomarker for the early detection of recurrence in resectable CRC, independent of clinico-pathological factors. Several Australian studies have reported that the ctDNA detection rate in patients with stage II colon cancer following surgery is 7.9%; while the ctDNA detection rate in patients with stage III colon cancer following surgery is 21% (18, 19). In a cohort of 486 patients with stage II-III colon cancers and locally advanced rectal cancer (LARC), patients with detectable ctDNA (MRD+ve) after surgery experienced lower 5-year recurrence-free survival (RFS) rates of 38.6% versus (vs) 85.5% ($P < 0.001$) and overall survival (OS) rates of 64.6% vs 89.4% ($P < 0.001$), when compared with those patients with undetectable ctDNA (MRD-ve) (20). In addition, the risk of recurrence is proportionately increased with higher levels of ctDNA VAF (hazard ratio [HR] = 1.2, 2.5 and 5.8 for VAFs of 0.1%, 0.5% and 1%, respectively) (20).

A Danish trial showed that the risk of post-operative recurrence increases if ctDNA becomes first detectable at the

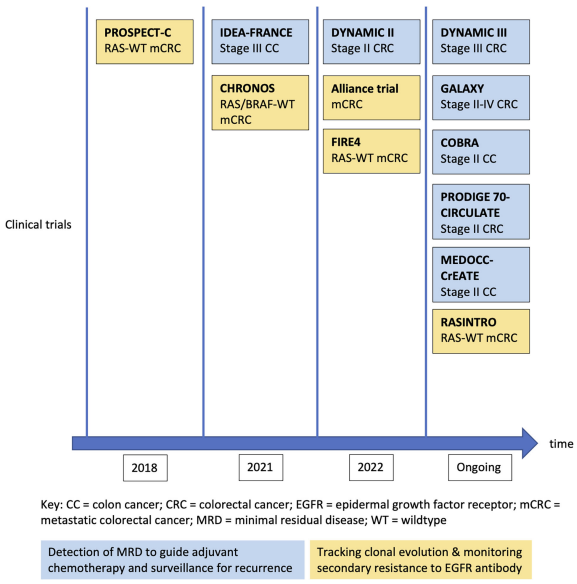


FIGURE 2
Development of major clinical trials for ctDNA application in colorectal cancers.

following time-points: 7 times higher risk if soon after surgery, 17.5 times if detected after adjuvant chemotherapy, and 43.5 times during surveillance (21). Interestingly, the duration of adjuvant chemotherapy may affect the prognostic significance of ctDNA (22). In a *post-hoc* analysis from the IDEA-FRANCE trial (which investigated the optimal duration of adjuvant chemotherapy by comparing 6 months vs 3 months of treatment in stage III colon cancer), ctDNA was prognostic in patients who received 3 months of adjuvant chemotherapy and with T4 and/or N2 tumors, but not in those treated for 6 months and with T1-3/N1 tumors (22).

Besides investigating the prognostic significance of postoperative ctDNA status, other studies compared the performance of ctDNA and radiological imaging in the detection of post-operative recurrence. However, the results are mixed because of the different imaging intervals used across different studies (21, 23, 24). A Danish study showed that checking ctDNA every 6 months could detect cancer recurrences up to 16.5 months earlier than radiologic imaging (21). This result is supported by a prospective study using the Signatera assay (23), where ctDNA could detect recurrences at a median of 9.08 months earlier than imaging in 193 patients with stage II-III CRC. In the TRACC study (25), post-operative MRD+ve status was the most powerful prognostic factor associated with increased RFS (HR = 28.8; 95% CI = 3.5 - 234.1; $P < 0.001$), compared with clinical factors, microsatellite (MSI) and tumor mutational burden (TMB) in 122 patients with stage II-III CRC. In contrast, a retrospective study (n = 48, Signatera assay) found no significant differences in the lead time or rate of detecting postoperative recurrence between ctDNA and imaging - the latter performed at intervals recommended by the United States (US) National Comprehensive Cancer Network (NCCN) guideline (24). The ongoing observational study (BESPOKE) will evaluate the impact of ctDNA testing (Signatera assay) on adjuvant treatment decisions and detection of recurrence in stage I-IV CRC across over 200 US sites (26). In conclusion, these observational studies have shown that ctDNA could accurately detect MRD status after surgery and predict disease recurrence, thus prospective randomized trials are warranted to determine if ctDNA will influence treatment decisions.

### 3.1.2 Recently reported phase III studies

There are at least 7 ongoing phase III studies with an interventional, observational or adaptive-platform design. These studies investigate the utility of postoperative MRD detection using ctDNA in guiding de-escalating or escalating adjuvant approaches in the management of stage II-III and/or resectable stage IV CRC (Table 1).

Several trials are investigating whether ctDNA-detected MRD can outperform conventional methods of directing adjuvant chemotherapy (Table 1). The DYNAMIC II (28) and III (ACTRN-12617001566325) studies are interventional studies led by the Australasian Gastro-Intestinal Trials Group for patients with stage II to III CRC. The pivotal DYNAMIC II trial is the first of these studies to be published recently, where 455 patients with stage II colon cancer (T3 or T4, N0, M0) were randomized in a 2:1 ratio to have treatment decisions guided by either ctDNA results (using Safe-Seq assay in a central laboratory) or standard clinicopathological features (29). Patients with a positive ctDNA result at either 4-week or 7-week after surgery received adjuvant fluoropyrimidine or oxaliplatin-based chemotherapy. Designed to detect non-inferiority between the 2 arms, the study met its primary endpoint by showing that ctDNA-guided approach was non-inferior to standard management (93.5% and 92.4% respectively; 95% CI = 4.1 - 6.2 [non-inferiority margin, −8.5% points]) in terms of 2-year RFS. Moreover, fewer patients in the ctDNA-guided arm received adjuvant chemotherapy (15% vs 28%; relative risk = 1.82; 95% CI = 1.25 - 2.65). This study is the first to show that ctDNA-guided approach to the management of stage II colon cancer could reduce the use of adjuvant chemotherapy use without compromising RFS. There are several other ongoing trials which investigate the utility of ctDNA in guiding adjuvant decisions. These include the PEGASUS study (NCT04259944, uses the LUNAR1 assay, Guardant Health) which has a novel, real-time adaptive design, where patients with stage II-III CRC will switch chemotherapy regimens based on the MRD status monitored at 3-month intervals (30). The US NRG-led COBRA (NCT04068103) is an escalation trial for stage II colon cancer, where MRD+ve patients will receive adjuvant chemotherapy, while MRD-ve patients will undergo surveillance alone (31). The French PRODIGE 70-CIRCULATE (NCT04120701) study will screen over 2600 patients with stage II CRC and randomize 198 MRD+ve patients post-surgery to either adjuvant FOLFOX (infusional 5-fluorouracil, leucovorin and oxaliplatin) for 6 months or observation. Similar to the DYNAMIC studies, the MEDOCC-CrEATE study will enroll 1320 stage II colon cancer patients without indication for adjuvant chemotherapy based on current practice guidelines, and randomize them into two possible interventional arms: ctDNA-uninformed (standard observation without adjuvant chemotherapy) vs ctDNA-informed (adjuvant CAPOX or observation, depending on MRD status) (29).

The largest study to date is the colossal 'CIRCULATE-Japan', an adaptive platform study which investigates the utility of ctDNA in MRD detection for patients with resectable stage II to IV CRC *via*. Eligible patients are first enrolled into an observational screening study (called GALAXY) and undergo ctDNA testing (Signatera assay) before treatment and at defined intervals after surgery. Each participant's ctDNA results are made available to the treating physicians to guide adjuvant treatment or enrollment into either one of two interventional phase III studies – the VEGA and ALTAIR (32). Preliminary result of the GALAXY study on the ctDNA dynamics of 1040

TABLE 1 Ongoing phase III or large observational studies or abstract-only reports on minimal residual disease.

| Study name & design. First author & reference | Study population | Sample size | Assay | Timepoints of ctDNA analysis | Primaryendpoint | Preliminary results reported in abstract form |
|---|---|---|---|---|---|---|
| TRACC (NCT04050345) Phase II/III. Anandappa G, et al. (25) | High risk stage II, stage III CRC | 107 | Tumor-informed Multiplex PCR (Signatera) | Before surgery or nCRT, <8 weeks and 3 months post-surgery. | 3-year DFS | -Baseline: 100/107 pts (93.4%) = ctDNA+ve<br>-After treatment: 14/107 pts (13%) = MRD +ve;<br>-6/14 pts (42.9%) MRD +ve relapsed vs 8/93 pts (8.6%) MRD-ve,<br>-ctDNA status most significant prognostic factor associated with RFS |
| GALAXY study (UMIN000039205) Prospective observational study. Shirasu H, et al. (27) | Stage II-III, resectable stage IV CRC | 1040 | Tumor-informed Multiplex PCR (Signatera) | Before surgery, 4,12,24,36,48,72,96 weeks post-surgery | DFS | -188/1040 pts (18%) MRD+ve at 4 weeks<br>1-year DFS 47.5% in MRD+ve pts, vs, 1-year DFS 92.7% in MRD-ve pts. |
| VEGA study (jRCT1031200006) Phase III, non-inferiority study | ctDNA-ve pts at 4 weeks after surgery, high-risk stage II, low risk stage III CRC | 1240 | Natera, Inc, (bespoke, mPCR-NGS) | Postoperative week 4, then 3months after completing adjuvant chemo | DFS in ctDNA-ve pts randomized to surgery alone vs adjuvant CAPOX | Not available |
| ALTAIR study (NCT04457297) Phase III | Stage II-III or resectable Stage IV CRC who remain ctDNA+ve within 3 months after surgery and had adjuvant chemo | 240 | Tumor -informed Multiplex PCR (Signatera) | Postoperative and monthly up to 3 months | DFS in ctDNA+ve pts despite prior adjuvant chemo, randomized to trifluidine/tipiracil or placebo | Not available |
| DYNAMIC-III (ACTRN-12617001566325) Phase II/III | Stage III CRC | 1000 | Safe-SeqS | Week 5 to 6 postoperatively, then at end of adjuvant chemo | RFS for ctDNA +ve cohort and ctDNA-ve cohort, disease managed with escalated (if ctDNA +ve) de-escalated treatment (if ctDNA -ve) | Not available |
| DYNAMIC-RECTAL (ACTRN-12617001560381) | Locally advanced rectal cancer | 408 | Safe-SeqS | Week 4 and 7 post-op | RFS for ctDNA and pathology- guided treatment and standard of care | Not available |
| MEDOCC-CrEATE (NL6281/NTR6455) Phase III | Stage II colon cancer | 1320 | PGDx elio$^{TM}$ | Immediate post-operatively in intervention arm, end of trial in control arm | Proportion of pts receiving adjuvant chemo when ctDNA+ve after surgery | Not available |
| PRODIGE 70 CIRCULATE (NCT04120701) Phase III | Resected stage II colon cancer | 1980 | ddPCR (2 methylated markers WIF1 and NPY) | ≥ 2 weeks and <8 week postoperatively | ctDNA +ve cohort: 3-year DFS | Not available |
| COBRA (NCT0406810) Phase II/III | Resected stage IIA colon cancer | 1408 | Guardant Health LUNAR model | Post-operatively | Phase II subset: clearance of ctDNA for ctDNA+ve pts at baseline with/without adjuvant chemo ≤ 6 months from baseline.<br>Phase III subset: RFS in ctDNA+ve cohort randomized to with/without adjuvant chemo | Not available |

*(Continued)*

TABLE 1 Continued

| Study name & design. First author & reference | Study population | Sample size | Assay | Timepoints of ctDNA analysis | Primaryendpoint | Preliminary results reported in abstract form |
|---|---|---|---|---|---|---|
| PEGASUS (NCT04259944) Phase II | Resected T4N0 or stage III colon cancer | 140 | Guardant LUNAR-1™ | 2-4 weeks after surgery, then 3 monthly or after each treatment | Number of post-surgery and post-adjuvant chemo false-negative cases after a double ctDNA-negative detection | Not available |
| CIRCULATE AIO-KRK-0217 (NCT04089631) Phase II | Stage II colon cancer | 4812 | Not reported | ≤ 5 weeks postoperatively | ctDNA+ve: DFS in pts randomized to surgery alone or adjuvant chemo | Not available |
| BESPOKE (NCT04264702) Case-control study | Stage I-IV colon cancer | 2000 | Tumor -informed Multiplex PCR (Signatera) | Serially sampling post operatively up to 2 years | Impact of ctDNA on adjuvant treatment decisions<br>Determine rate of recurrence of pts diagnosed with CRC while asymptomatic using ctDNA | Not available |

CRC, colorectal cancer; PCR, polymerase chain reaction; DFS, disease free survival; MRD, minimal residual disease; +, positive; -, negative; RFS, relapse or recurrence-free survival, ddPCR, digital droplet polymerase chain reaction; Pt, patients; nCRT, neoadjuvant chemoradiotherapy; chemo, chemotherapy.

(out of a target of 5200) patients have been reported (27, 33). For all stages of CRC, patients who were MRD+ve at 4 weeks post-surgery (18%, 188/1040 patients) had 1-year disease-free survival (DFS) rate of 47.5%; while patients who were MRD-ve at 4 weeks post-surgery (82%, 852/1040 patients) had a 1-year DFS rate of 92.7% with a HR of 10.9 (95% CI = 7.8 - 15.4; P < 0.001). Adjuvant chemotherapy may be able to convert patients who were initially MRD+ve into MRD-ve. The use of adjuvant chemotherapy resulted in a higher proportion of patients (68%) being converted to MRD-ve at 12-weeks post-surgery, while only 10% of patients who did not receive chemotherapy were converted into MRD-ve. Patients who were successfully converted from MRD+ve to MRD-ve status had better DFS. In contrast, in patient subgroups who were MRD-ve at 4 weeks post-surgery, the use of adjuvant chemotherapy did not influence the DFS (HR = 1.3; 95% CI = 0.5 - 3.6%). In conclusion, this study supports the use of ctDNA at 4 weeks post-surgery to guide adjuvant therapy. The ongoing ALTAIR study will randomize patients who are ctDNA+ve at any time-point within 2 years after curative-intent surgery, to 6 months of oral trifluridine/tipiracil or placebo. The VEGA trial investigates a de-escalation strategy of randomizing patients who were MRD-ve at 4 weeks post-surgery to either surgery alone or 3 months of adjuvant CAPOX.

## 3.2 Use of ctDNA in guiding treatment of locally advanced rectal cancer during preoperative chemo-radiotherapy

LARC is usually treated with multimodality treatment with either concurrent chemoradiotherapy (chemo-RT) or total neoadjuvant therapy (TNT) followed by total mesorectal excision surgery (TME). In general, ctDNA can be detected in approximately 57 - 77% of patients before surgery, 15.6 - 22.3% of patients after neoadjuvant therapy and 10.5 - 12% of patients after surgery - allowing for differences in the patients and assays across studies (34–36). Sampling of ctDNA at any of these perioperative time-points are also prognostic to a different extent. Appelt et al. (37) found that patients with baseline detectable hypermethylated ctDNA predicted improved OS (HR = 2.08; 95% CI = 1.23 - 1.51) and freedom from distant metastases (HR = 2.20; 95% CI = 1.19 - 4.07). Zhou et al. (34) showed that median VAF in baseline ctDNA was a strong independent predictor of metastasis-free survival (MFS) (HR = 1.27; P < 0.001). However, another study showed no association between ctDNA at baseline and MFS (38).

There is increasing interest in organ preservation strategies to spare patients the morbidity of a TME surgery. The recently reported phase II OPRA trial (NCT02008656) showed that up to half of patients may be able to achieve a clinical complete response (cCR) with a TNT approach without a detriment to DFS. ctDNA may complement conventional approach of predicting response to neoadjuvant therapy. Wang et al. (39) constructed a risk model unifying baseline ctDNA, ctDNA clearance, tumoral mutation status and magnetic resonance imaging (MRI)-based tumor regression grade in the prediction of pathological complete remission (pCR) after neoadjuvant therapy. This model was shown to be more accurate than models that were derived from only ctDNA or only MRI-based tumor regression grade.

The role of adjuvant chemotherapy after chemo-RT and surgery is controversial, with only one study showing a progression-free survival (PFS) benefit with FOLFOX in stage III LARC (40). Patients with detectable ctDNA after surgery are significantly associated with worse RFS if ctDNA is detected as early as 4-6 weeks after neoadjuvant chemo-RT (HR = 6.6; P < 0.001) or as late as 4-10 weeks after surgery (HR = 13.0; P < 0.001).

This prognostic significance of MRD+ve status is independent of adjuvant chemotherapy and clinicopathological risk factors (36). This finding has formed the basis of the ongoing DYNAMIC RECTAL study (ACTRN-12617001560381) which will randomize patients to chemotherapy or surveillance after surgery, depending on the MRD status.

In conclusion, ctDNA has the potential of directing adjuvant therapy and improving the accuracy of assessing response to neoadjuvant therapy. These in turn may help to identify patients who might be candidates for de-escalated approaches, such as surveillance alone after TME, sphincter-preserving surgery or a wait-and-watch approach without surgery after chemo-RT. Validation in larger prospective trials using a risk-adapted approach in the management of LARC are ongoing.

## 3.3 Monitoring response to palliative chemotherapy in metastatic colorectal cancer

In stage 4 CRC, ctDNA VAF is significantly associated with the number of metastatic sites (41) and is prognostic in resectable or unresectable metastatic CRC (42). Chemotherapy and targeted therapy are part of the standard treatments for stage 4 CRC, and various biomarker-guided therapies targeting *BRAF* mutation and *EGFR*-mediated signaling have improved patient survival (3). Many studies have reported a high concordance between tumor and plasma in the detection of *KRAS* and *BRAF* mutations using ddPCR (43, 44). For patients with resectable oligometastatic CRC, detectable levels of ctDNA after surgery and/or post-operative chemotherapy are associated with shorter RFS (45).

The dynamic changes of ctDNA during the first few cycles of chemotherapy may predict radiologic response. In a study by Tie et al., 74% of patients had a 10-fold decrease in ctDNA level before cycle 2, which correlated with radiologic responses at 8-10 weeks (odds ratio [OR] = 5.25; 95% CI = 1.38 – 19.93; P = 0.016) (46). Conversely, an increase in ctDNA during the first cycle of chemotherapy could predict inferior outcome (47). The findings by Tie et al. are supported by another prospective study by Garlan et al., where 'ctDNA responders' had superior radiologic response, PFS and OS than those who were ctDNA non-responders (48).

## 3.4 Tracking clonal evolution and monitoring secondary resistance to epidermal growth factor receptor (*EGFR*) antibody

### 3.4.1 Clonal dynamics during *EGFR* therapy alone or in combination with chemotherapy

In clinical practice, *RAS* and *BRAF* mutations are routinely analyzed to guide anti-*EGFR* therapy in mCRC. Other molecular alterations also contribute to resistance to *EGFR* antibody therapy, such as *PIK3CA* mutation, *HER2*, *MET* and *ERBB2* amplifications (49–51). Several landmark studies have suggested that when cancer cells are subjected to therapeutic pressure during anti-*EGFR* therapy, they acquire secondary genetic alterations in a process known as clonal evolution, which may contribute to drug resistance. Emergence of resistant clones can be tracked serially using ctDNA during anti-*EGFR* therapy, at as early as 10 months before the overt development of clinical resistance (52). Diaz et al. suggested that these drug-resistant *KRAS* mutant cancer cells are already present before a patients is started on *EGFR* antibody treatment (53). To confirm these findings, the PROSPECT-C phase II study was carried out to track the clonal evolution of resistant subclones using ctDNA during *EGFR* antibody therapy in patients with *RAS*-wildtype (WT) CRC. At baseline, 50% of patients already harbored aberrations in *RAS* pathway and *BRAF V600E* mutations in their ctDNA (54), and most patients (86.3%) would have detectable ctDNA levels of these resistant mutations at clinical progression (54).

ctDNA has also been used to track clonal evolution in treatment-naïve patients receiving *EGFR* antibodies together with chemotherapy. The CALGB/SWOG 80405 (Alliance) (55) is a first line study which randomizes patients to two different drug sequences of first-line therapy for stage IV CRC: chemotherapy plus *EGFR* antibody (cetuximab) or chemotherapy plus *VEGF* antibody (bevacizumab). In the *post-hoc* analysis of 133 patients with *RAS/BRAF*-WT CRC, ctDNA tracking showed a trend towards a higher prevalence of acquired mutations associated with resistance to *EGFR* antibody in patients (n = 11; 15.3%) randomized to the bevacizumab arm than patients in the cetuximab arm (n = 5; 8.2%) (OR = 2.0; P = 0.29). These provocative findings seem to suggest that exposure to bevacizumab in the first-line setting may increase the chance of patients acquiring *EGFR* antibody resistance-associated genomic alteration, thus further validation is warranted.

### 3.4.2 *EGFR* antibody rechallenge

ctDNA is useful in selecting patients for *EGFR* antibody rechallenge. Clonal evolution is a dynamic process and therefore the optimal time-point and ctDNA VAF thresholds for determining whether a patient could be re-challenged with *EGFR* antibody therapy need to be defined. Siravegna et al. reported that the circulating level of mutant *RAS* clones increase initially during anti-*EGFR* therapy and gradually fall when therapy was withdrawn (49). Furthermore, circulating *RAS* and *EGFR* VAF undergo exponential decay at cessation of *EGFR* antibody therapy with a cumulative half-life of around 4.4 months (56). In a cohort of 80 patients who were re-treated with *EGFR* antibody, an overall response rate (ORR) of 23% was observed. A non-statistical trend towards higher ORR and PFS was noted if these patients were re-challenged after a longer drug

holiday (in terms of <1 vs 2 half-lives) from the last *EGFR* antibody therapy (56). This knowledge may provide insight into the optimal timing of *EGFR* antibody re-challenge, however, the most appropriate VAF threshold that can guide treatment remains unclear (16). A meta-analysis showed that in patients without detectable *RAS* mutation in ctDNA, re-challenge with *EGFR* antibody therapy was associated with a larger benefit in PFS (HR = 0.40; 95% CI = 0.22 - 0.70; P = 0.001) and OS (HR = 0.37; 95% CI = 0.16 - 0.85; P = 0.02) than in patients with detectable *RAS* mutation (57). Several trials investigating the clinical impact of ctDNA-guided re-challenge of *EGFR* antibody therapy are ongoing, these include the RASINTRO (NCT03259009), FIRE4 trial (58) and the CHRONOS study (59) (Table 2). The CHRONOS is an interventional study which enrolls responders to *EGFR* antibody therapy who are 'triple wild-type' in *RAS*, *BRAF* and *EGFR* ectodomain in ctDNA. Patients will be re-challenged with panitumumab while ddPCR and NGS are used to track clonal evolution. Of the 52 patients screened in a preliminary report, 36 (69%) were triple wild-type, 27 received panitumumab with an ORR of 30% (59).

### 3.4.3 Other targeted therapies

For other rarer molecular subgroups such as *BRAF* mutant, *HER2* amplified and MSI-high (MSI-H) CRC, newer drug therapies are becoming available in the clinic (3). ctDNA monitoring has been used to track clonal evolutions in patient subgroups with *BRAF V600E* mutations and *HER2* alterations in some clinical trials. In an exploratory analysis of the phase III BEACON trial (60) which demonstrated the superiority of targeting *BRAF-EGFR-MEK* inhibition with encorafenib-binimetinib-cetuximab over cetuximab-chemotherapy, over 90% of patients had detectable *BRAF V600E* mutations in ctDNA (GuardantOMNI assay). ctDNA VAF was found to be prognostic but not predictive of drug response (61). Patients with low ctDNA VAF (defined as lower than the median VAF) had longer median OS (14.8 months; 95% CI = 11.7 – 23.0) than in those with higher VAF (5.4 months; 95% CI = 4.4 – 6.1) when treated with encorafenib-cetuximab (61). In the phase II TRIUMPH trial (UMIN000027887) which tested the combination of trastuzumab plus pertuzumab in patients with *HER2*-amplified stage IV CRC, both tissue and ctDNA were used for determining *HER2* status. The ORR were similar in patients who were tested *HER2*+ve with tissue compared with ctDNA (ORR = 30% vs 28% respectively) (62).The COLOMATE study (NCT03765736) is an ongoing, seamless adaptive protocol that primarily uses ctDNA (Guardant 360) to screen patients with secondary resistance to targeted therapies, for enrolment into 3 different clinical trials depending on their ctDNA genotype: panitumumab re-challenge (PULSE study NCT03992456); tucatinib, trastuzumab, and TAS-102 for patients if ctDNA show *HER2*-alteration; and re-challenge with encorafenib, cetuximab and binimetinib if patient is *BRAF V600E*-mutant (63).

## 4 Gastric and esophageal cancer

### 4.1 Gastric and gastroesophageal junction adenocarcinoma

The application of ctDNA in gastric cancers (GC) remains challenging due to the relatively low frequency of genomic alterations, the larger inter-patient and intra-patient temporo-spatial heterogeneity in tumors and plasma, as well as impaired

TABLE 2 Studies evaluating circulating tumor DNA as a screening tool to detect patients who could benefit from EGFR antibody re-challenge in metastatic colorectal cancer.

| Study name | Study design | Estimated sample size | Assessment method/ Assay | Mutation analyzed | Primary outcome | Secondary outcome | Results (abstract only) |
|---|---|---|---|---|---|---|---|
| CHRONOS (NCT03227926) Sartore-Bianchi A, et al. (59) | Phase II RCT | 52 | ddPCR, NGS | *RAS/EGFR/ BRAF* | ORR | PFS, OS, Toxicity | -36/52 pts (69%) negative for *RAS/BRAF/ EGFR* mutations. -ORR for rechallenge EGFR antibody = 30% |
| RASINTRO (NCT03259009) | Prospective observational cohort | 73 | NGS | *RAS* | PFS | Tumor response, OS | Not available |
| FIRE-4 (NCT02934529) | Phase III RCT | 550 | Not available | *RAS* | OS | PFS, ORR, molecular biomarker | Not available |
| PULSE (NCT03992456) | Phase II RCT | 120 | NGS (Guardant 360) | *RAS* | OS | PFS, ORR, CBR | Not available |
| COLOMATE (NCT03765736) | Prospective observational cohort | 500 | NGS (Guardant 360) | RAS/ERBB2/ BRAF | Proportion of patients with an actionable genomic profile | Not available | Not available |

RCT, randomised controlled trial; ddPCR, digital droplet polymerase chain reaction; NGS, next generation sequencing; ORR, overall response rate; PFS, progression free survival, OS, overall survival; CI, confidence interval; CBR, clinical benefit rate.

tumor shredding from peritoneal metastases (64–66). Around 37% of GC tumors contain actionable somatic mutations (e.g. *KRAS*, *TP53*, *PIK3CA*) or gene amplifications (e.g. *HER2*, *MET*, *EGFR*, *FGFR2*, *ERBB2*) (64, 67–69). Ichikawa et al. found that 68.1% of cancer-related genes identified in ctDNA of patients with GC are actionable, with *TP53* mutation and *ERBB2* being the most common (70). Maron et al. reported that in a large cohort over 1600 patients with GEA, the presence of some actionable *RTK* amplifications (e.g. *HER2*, *EGFR*, *MET*, *FGFR2*) are of prognostic significance (64).

Several comparative analyses of genomic profiling using ctDNA and tumors have been carried out in GC. The Korean VIKTORY trial of stage IV GC reported a 89.5% concordance between liquid and tumor biopsy for *MET* amplification (71). Schrock et al. reported a 86% concordance in genomic alterations detected in tissue and plasma derived from 417 patients with GI cancers; however, only 63% of alterations found in ctDNA were detected in tumor, suggesting intra-tumoral heterogeneity (69). Moreover, the concordance rate was lower (50%) for gene amplifications such as *HER2*. Studies on the concordance between *HER2* amplification in tumors using conventional methods (immunohistochemistry [IHC], or FISH) and ctDNA have shown mixed results. Some studies found high concordance with ddPCR (72, 73), but another showed that only 62% of patients with known *HER2*+ve tumors had detectable *HER2* amplification in ctDNA (64). In conclusion, these studies suggest that genotype information from ctDNA is complementary but cannot replace tumor-based NGS in GC (64).

### 4.1.1 Minimal residual disease detection post-surgery

Similar to CRC, ctDNA has been investigated in the detection of MRD detection in resectable GC, gastroesophageal junction (GEJ) and esophageal adenocarcinoma (EAC). Data are limited by the relatively low level of ctDNA found before surgery (42 - 47%) in GC or EAC (74, 75). In one of the largest study in GEA, Maron et al. evaluated the utility of a commercial ctDNA-NGS assay (Guardant 360) 1630 patients with GC and EACs. MRD detection after curative surgery of EACs is strongly associated with an increased risk of recurrence (64). Kim et al. found that postoperative MRD+ve status in stage I-III GC precedes radiographic progression by 6 months (76), and is associated with shorter DFS (HR = 14.78; 95% CI = 7.991 – 61.29; P < 0.0001) and OS (HR = 7.664; 95% CI = 2.916 – 21.06; P = 0.002) (75). Similar findings are also reported by Openshaw et al. in GEJ cancers with shorter RFS (HR = 3.7; P = 0.028) (77).

### 4.1.2 CtDNA in patients with advanced gastric cancer undergoing systemic therapy

In patients with advanced GC undergoing systemic therapy, Maron et al. showed that the maximal tumor VAF (maxVAF) in ctDNA could reflect tumor burden, such that in patients with a baseline maxVAF level of > 0.5%, who experienced a ≥ 50% fall in the maxVAF level during the first 5 months of systemic treatment, had superior median OS of 13.7 vs 8.6 months than those who had not (HR = 0.3; 95% CI = 0.1 – 0.8; P = 0.02) (64). The role of ctDNA in tracking clonal evolution in patients undergoing trastuzumab or lapatinib-based therapy has been evaluated in another study, where ctDNA monitoring has revealed multiple alterations that are purportedly associated with secondary resistance to anti-*HER2* therapies, such as *MYC*, *EGFR*, *FGFR2* and *MET* amplifications (78), as well as *PIK3CA*, *ERBB2/4*, *NF1* and *KRAS Q61R* mutations (79, 80).

The PANGEA is the first reported prospective study using a biomarker-guided platform to individualize patients with stage IV GEA for systemic therapy (81). Pre-treatment tumor and ctDNA-based NGS target sequencing, IHC of programmed death receptor-1 ligand (PD-L1) expression, TMB and Epstein-Barr virus (EBV) status were used to stratify and assign patients to receive 1 out of 6 matched monoclonal antibody against *PD1*, *EGFR*, *HER2*, *FGFR2* or *VEGFR2* (81). The PANGEA met its primary endpoint with 45 of 68 (66%) patients alive at 12 months - exceeding the 50% historical control rate (81). The PLAGAST (NCT02674373) study is an ongoing non-interventional study which is aimed at evaluating the association of ctDNA dynamics with prognosis and response in patients with GC undergoing systemic therapy. The Oesophageal Cancer Clinical Molecular Stratification (OCCAMS) Consortium is leading an ongoing study of patients with resectable EAC where ctDNA will be performed (Signatera assay) during postoperative surveillance. A preliminary report on 12 patients showed that MRD+ve has a sensitivity and specificity of 100% in detecting early postoperative recurrence (82). Ococks et al. reported that ctDNA+ve patients have a longer median cancer-specific survival (10.0 months) than ctDNA negative patients (29.9 months) (HR = 5.55; 95% CI = 2.42 - 12.71; P = 0.0003) (83). Bonazzi et al. reported that detectable ctDNA variants in post-treatment plasma is associated with inferior disease-specific survival, and VAF increased with recurrence (84). In conclusion, these studies validate that ctDNA is prognostic for relapse and survival, and could be incorporated for risk stratification of patients for adjuvant chemotherapy escalation or de-escalation.

## 4.2 Esophageal squamous cell cancer (ESCC)

The mutational profile of ESCC is different from that of esophageal adenocarcinomas (EAC), but similar to that of other squamous cell cancers (85, 86). In a meta-analysis on sequencing methodologies including ctDNA analysis in ESCC, ctDNA assays have a relatively low sensitivity of 48.9% (29.4 - 68.8%), but high specificity of 95.5% (90.6 - 97.9%) for detecting recurrence post-

surgery (87). The data on the utility of ctDNA in MRD detection in ESCC are mostly derived from small, retrospective studies. Two reports reported a decrease in ctDNA VAF in patients post-surgery (88, 89). In patients with localized ESCC undergoing neoadjuvant therapy, MRD+ve status post-treatment was associated with increased risk of tumor progression (HR = 18.7; P < 0.0001), distant metastases (HR = 32.1; P < 0.0001) and shorter disease-specific survival (HR = 23.1; P < 0. 0001) (14).

# 5 Pancreatico-biliary cancer

## 5.1 Pancreatic ductal adenocarcinoma (PC)

The genomic characterization of pancreatic cancer (PC) shows that somatic mutations of *KRAS*, *TP53*, *CDKN2A* are common (90). In a meta-analysis on 369 patients, *KRAS* mutation can be detected in ctDNA with a pooled sensitivity of 70% and specificity of 86% (91). However, one of the major limitations on the clinical applicability of ctDNA-NGS in PC is the low concordance rate of 31.9% in the tumor vs ctDNA-derived result (91). This may be due to the hepatic clearance of ctDNA released from the PC primary at the hepatic portal vein (92). Another limitation is the false-positive ddPCR results caused by benign conditions such as pancreatitis, therefore the additional use of methylation markers has been suggested to minimize this possibility (92, 93).

The prognostic value of ctDNA has been evaluated in a recent meta-analysis of 48 studies of over 3000 patients with different stages of PC. This study found that the detection of *KRAS* mutations *via* ctDNA has a negative impact on OS and PFS in PC (HR = 2.42; 95% CI = 1.95 - 2.99 and HR = 2.46; 95% CI = 2.01 - 3.00, respectively) (94). In localized PC, detection of ctDNA preoperatively is associated with poorer RFS (HR = 4.1; P = 0.002) and OS (HR = 4.0; P = 0.003) (95). This is consistent in another study, where ctDNA detection is associated with inferior RFS and PFS (HR = 2.27; 95% CI = 1.59 – 3.24; P < 0.001) and OS (HR = 2.04; 95% CI = 1.29 – 3.21; P = 0.002) (96). These studies suggest that MRD detection using ctDNA in the early postoperative period is prognostic in resectable PC (95, 97, 98), but may be affected by the use of neoadjuvant chemotherapy (99). Nevertheless, subsequent detection of ctDNA during surveillance strongly predicts recurrence with a 90% sensitivity and 88% specificity (99). The ongoing interventional phase III DYNAMIC-Pancreas study (ACTRN-12618000335291) in early-stage PC will evaluate the utility of ctDNA in guiding adjuvant therapy in resectable PC.

Most studies which investigated the potential of ctDNA in monitoring response to chemotherapy in advanced PC used *KRAS* genotyping, while a few targeted other clonal mutations. *KRAS* mutation can be detected in 36 out of 54 (67%) of patients with advanced PC (100). Collectively, several studies have shown that ctDNA increase tends to precede clinical progression as determined by imaging and serum Ca19.9 level by a few months (100–102). In a meta-analysis of studies on patients with detectable *KRAS* before treatment, conversion to undetectable *KRAS* after treatment is associated with better prognosis (94). ctDNA has also been used to track other cancer-specific mutations such as *TP53*, *APC*, *ATM*, *FBXW7*, *SMAD4*, *CDKN2A* and other variants (101, 103). *BRCA1/2* mutations can be found between 1-10% of PC and may predict response to PARP inhibitors in the palliative setting (104). A study has found a high degree of concordance in *BRCA* mutation detected in tissue and plasma (103). Larger studies are needed to test the feasibility of using ctDNA to select and monitor patients for *BRCA1/2* mutation and PARP inhibitor therapy. In conclusion, the development of ctDNA in monitoring response to systemic treatment is still at an early stage and requires further validation.

## 5.2 Biliary cancer - extrahepatic (EHCC), intrahepatic (IHCC) and gallbladder cancer

Most patients with cholangiocarcinomas (CC) - including IHCC, EHCC and gallbladder cancer, are usually diagnosed at an advanced stage where post-operative recurrence risk is high (105). *FGFR1-3* fusions and *IDH1/2* mutations can be found in 15 - 20% of IHCC, where the concordance of tumor and ctDNA-derived is higher for IHCC (92%) than that of EHCC (55%) (105). The detection of ctDNA using target-panel NGS has been used to track clonal evolution during chemotherapy, demonstrating that over 60% of patients may develop new driver genes at progression (105). There have been significant advances in the development of new targeted therapies for CC such as *IDH1* inhibitor for *IDH1* mutant tumors (106), and *FGFR* inhibitors for tumors harboring *FGFR2* fusions (107, 108). ctDNA has been investigated in selecting patients for such therapies and in tracking emergence of secondary resistance to these agents. Goyal et al. were the first to describe the molecular basis of acquired resistance to a *FGFR2* antibody (BGJ39) (109) by using serial cfDNA monitoring during treatment. An acquired *V564F* mutation was found in 3 out of 4 patients who progressed, while 2 progressors had multiple *FGFR* point mutations. There was a high concordance between tissue and ctDNA in detecting these resistant variants. This study may pave the way for larger studies on ctDNA in guiding anti-*FGFR2* therapy for CC.

# 6 Response monitoring of immune-checkpoint inhibitor therapy in GI cancers

Immune-checkpoint inhibitors (ICI) such as PD1 and CTLA-4 therapy are now part of the standard therapeutic options for stage IV MSI-H CRC in the first and subsequent line settings. In addition,

patients with other GI cancers that are MSI-H or TMB > 10 mut/Mb (110) may be suitable for anti-PD1 therapy in the palliative setting. Several studies have investigated the feasibility of ctDNA in assessing MSI, TMB status in GI cancers. Nakamura et al. compared ctDNA NGS (Guardant 360) and tissue based MSI assessments in a cohort of 658 patients with advanced GI cancer in the SCRUM-Japan GOZILA study - an observational ctDNA-based study which screens patients with GI cancers for enrollment into clinical trials within a nation-wide trial network (111). The concordance between tumor and ctDNA for detection of MSI is high with an overall percent agreement of 98.2% (95% CI = 96.8 - 99.1). In particular, ctDNA was able to identify patients with MSI-high tumors who might benefit from anti-PD1 therapy (111). Using the Guardant360 assay, Maron et al. reported a 100% concordance between tumor-derived MMR status (IHC) and plasma-derived MSI-status using ctDNA-NGS in 6 patients (64). In contrast, there is significant discordance between tumor and ctDNA-derived TMB assessment. In a 'real-life' retrospective study of 410 patients (82 had GI cancers) with matching TMB results from tumor and plasma-based commercial NGS assays in the community setting, the median TMB was higher in plasma ($m$ = 10.5 mut/Mb) than in tumor ($m$ = 6.0 mut/Mb; P < 0.001). This will have obvious implication on selecting patients with non-CRC GI cancers for PD1 inhibitors based on ctDNA TMB alone, since the drug label for the tissue-agnostic approval of pembrolizumab recommends that the TMB threshold should be ≥ 10 mut/mb. In conclusion, if ctDNA TMB is used to select patients with GI cancers for PD1 inhibitor, a much higher ctDNA TMB threshold (up to 12 to 40 mut/Mb depending on the assays used) should be used to guide treatment decisions (112).

The monitoring of ctDNA during ICI therapy has been investigated in stage IV MSI-H CRC in a number of small cases reports. In these studies, the following endpoints were analyzed: the quantitation of VAF, measurement of TMB and tracking of tumor-specific mutations such as *TP53*, *RAS* and *BRAF* (113). Some studies have suggested that MSI-H CRC are often poorly-differentiated and produce significantly lower levels of serum tumor markers such as CEA and CA 19.9 (114–116) than well-differentiated tumors. Therefore, ctDNA holds promise as a blood-based predictive biomarker of response to ICI for such patients.

In Zhang et al.'s study of 978 patients across 16 tumor types (48 had GEAs, 32 had PC and 58 had MSI-H solid tumors) who were undergoing ICI therapy, changes in VAF during treatment could predict drug response, such that patients who could completely clear ctDNA (VAF = 0) had longer PFS and OS (P < 0.0001) than those who could not (117). Similarly, Kim et al. also found that in a study of 61 patients with stage IV GC treated with a PD1 inhibitor, changes in the ctDNA levels at 6 weeks post-treatment correlated with PFS and ORR (118).

Apart from VAF clearance as an endpoint, another study by Jin et al. investigated other endpoints e.g. 'decline in maxVAF' and 'ctDNA-positivity' *via* a NGS ctDNA assay, in 46 patients treated with PD1 inhibitor alone or in combination with chemotherapy (119). The median PFS was significantly longer in patients who experienced > 25% decline in maxVAF (7.3 months vs 3.6 months, P = 0.0011; 53.3% vs 13.3%, P = 0.06), and in those who had undetectable ctDNA (7.4 months vs. 4.9 months, P = 0.025) after ICI-based therapy (119).

## 7 Current challenges and future directions

Advances in NGS and PCR technologies have enabled the accurate detection and quantification of ctDNA in patients with different stages of GI cancers. There is a practical need to identify an informative and less invasive biomarker to help guide adjuvant, neoadjuvant and palliative drug therapies. The strongest evidence available to date showed that ctDNA is a strong prognostic marker when used to detect MRD following curative intent surgery in resectable GI cancer. The DYNAMIC II study is the first to show that ctDNA can direct adjuvant chemotherapy in the management of stage II colon cancer without compromising RFS. Several interventional studies with adaptive design using ctDNA as a screening platform are ongoing in patients with resectable CRC, PC and LARC. These studies are designed to definitively address the questions of whether ctDNA is superior to conventional methods of guiding adjuvant chemotherapy on patient's survival, and whether ctDNA guided escalation or de-escalation of adjuvant therapy may help to improve survival and minimize the risk of long-term treatment-related morbidities.

In the palliative setting, there are emerging data to suggest that ctDNA dynamics during the early treatment period are both prognostic and/or predictive of subsequent response to systemic treatments. Furthermore, serial measurement of ctDNA during targeted therapies has enabled tracking of clonal evolution and emergence of secondary resistance-related variants to some targeted therapies. Some evidence supports the use of ctDNA in guiding *EGFR* antibody rechallenge followed by ICI, while more evidence is needed for other targeted therapies e.g. *HER2* or *BRAF*.

As NGS technologies and other pre-analyzed variables are refined continuously with time, the cost and accuracy of ctDNA are likely to improve with time. There remain challenges that need to be overcome. It is unclear whether it is more informative to do both blood and tumor NGS at baseline than other modality alone in guiding treatment decisions or in selecting patients for clinical trials, given the intratumoral heterogeneity in GI cancers. Furthermore, there is a lack in consensus on determining the most biologically meaningful thresholds of ctDNA-related metrics (e.g. maxVAF, percentage change in VAF) to guide oncologists in practice. In postoperative surveillance, it is unlikely that ctDNA will completely replace conventional diagnostic and staging tools e.g. imaging and protein-based

serum cancer markers in the management of GI cancers. In clinical trials, ctDNA may potentially accelerate drug development by facilitating the molecular genotyping of patients for clinical trials of novel targeted therapies, in detecting early signals of drug response and in tracking emerging clonal resistance (120).

In conclusion, it is important to reach consensus on how ctDNA as a biomarker should be practically incorporated into current complex treatment algorithms to guide the treatment of GI cancers in potentially curative and palliative settings. One of the possible directions is to use the massive volume of genomic data derived from the systemic profiling of ctDNA for the development of artificial intelligence driven computational models and programs that can be applied in the routine oncological care of patients with GI cancers.

## Author contributions

RL and BM are responsible for overall design, planning, writing and editing of manuscript and figure. DJ is responsible for writing up of specific sections and the table. WL, KC, GL, ML, and JW are responsible for planning and editing of specific sections. All authors contributed to the article and approved the submitted version.

## Funding

This review is supported in part by the Kingboard Precision Oncology Program and the Charlie Lee Precision Immuno-oncology program, The Chinese University of Hong Kong, Hong Kong SAR.

## Acknowledgments

We would like to thank Ms. Alice Kong for clerical assistance.

## Conflict of interest

KC is a director of Take2, DRA and Novostics. KC holds equities in Take2, DRA, Grail/Illumina. KC and WL were previous consultants to Grail. WL holds equity in Grail/Illumina. KC holds patents portfolio in molecular diagnostics and receive royalties from Take2, DRA, Grail, Illumina, Sequenome, Xcelom.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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frontiers | Frontiers in Oncology

TYPE Case Report
PUBLISHED 28 November 2022
DOI 10.3389/fonc.2022.944963



OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology

RECEIVED 16 May 2022
ACCEPTED 07 November 2022
PUBLISHED 28 November 2022

CITATION
Bai Y, Yu Q, Liu N, Liu J, Wang D, Liu X and Yuan S (2022) Case report: Cerebrospinal fluid-derived circulating tumor DNA diagnoses and guides the treatment of a lung adenocarcinoma case with leptomeningeal metastasis. *Front. Oncol.* 12:944963. doi: 10.3389/fonc.2022.944963



# Case report: Cerebrospinal fluid-derived circulating tumor DNA diagnoses and guides the treatment of a lung adenocarcinoma case with leptomeningeal metastasis

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Leptomeningeal metastasis (LM) occurs in 3~5% of non-small cell lung cancer (NSCLC) patients. Diagnosis of patients with LM and disease monitoring remains challenging due to the low sensitivity and specificity of the commonly used approaches, such as cerebrospinal fluid (CSF) cytology and magnetic resonance imaging (MRI). Therefore, new approaches are necessary to improve the detection of LM. Recent studies have shown that circulating tumor DNA (ctDNA) in CSF can be used to detect and monitor LM, but whether it can serve as an early diagnostic biomarker prior to cytological and radiographic evidence of LM involvement requires further evaluation. Here we report a lung adenocarcinoma patient who had detectable oncogenic mutations in the CSF ctDNA prior to confirmation of LM by CSF cytology and MRI, highlighting the potential application of CSF ctDNA in early detection of LM.

KEYWORDS

**NSCLC, ctDNA, leptomeningeal metastasis (LM), cerebrospinal fluid (CSF), case report**

## Introduction

Leptomeningeal metastasis (LM) can be found in 3~5% of NSCLC patients (1). The diagnosis of LM is usually based on the clinical manifestations and a combination of cerebrospinal fluid (CSF) cytology and neuroimaging characteristics. Although CSF cytology remains the gold standard for LM detection, the sensitivity is only 50% (2). Magnetic resonance imaging (MRI) of the brain and spine is also a conventional technique for valuating LM. However, it has been reported that 20-30% of patients with confirmed LM had a false-negative MRI (3, 4). Therefore, it is crucial to identify new approaches to improve the diagnosis and characterization of LM in NSCLC patients. Recent studies have shown that circulating tumor DNA (ctDNA) in CSF can be used to characterize and monitor LM (5), but whether it can function as an early diagnostic biomarker prior to cytological and/or radiographic evidence of LM spread requires further evaluation. In this case, we report the presence of oncogenic mutations in the CSF ctDNA earlier than cytology and MRI-confirmed LM in a lung adenocarcinoma patient and discuss the potential application of CSF ctDNA in the early detection of LM.

## Case report

A 55-year-old female never-smoker was admitted to the hospital in August 2019 with a pulmonary mass detected by chest computed tomography (CT) during physical examination (Figure 1A). Her main complaint was right scapular pain, with a numerical rating scale score of 3. The patient had no other significant pulmonary symptoms, such as cough, tachypnea, or respiratory distress. Further pathological examination of the biopsy of the right lung confirmed lung adenocarcinoma (LADC). Subsequent radionuclide bone scan and abdominal CT revealed multiple bone and liver metastases, respectively. Thus, the patient was diagnosed with stage IVb LADC. To identify potential treatment options, the primary lung tumor biopsy and whole blood normal control were subjected to next-generation sequencing (NGS) tests. While waiting for the test results, the patient was treated with radiotherapy (48Gy/16fractions) to the right scapula targeting the bone metastasis, in combination with one course of chemotherapy (pemetrexed 0.8g d1+carboplatin 0.4g d2). NGS tests revealed a classic epidermal growth factor receptor (*EGFR*) exon 19 (*EGFR* 19Del) in-frame deletion (Table 1). Thus, an EGFR-targeted therapy, gefitinib (0.25g qd), was administered. However, ten days following diagnosis, the patient developed severe headache and nausea, which was accompanied by aphasia, restlessness in the limbs, and a sudden vision loss in both eyes, suggestive of brain abnormality. CT scan of the brain showed no obvious abnormality. We also obtained cerebrospinal fluid (CSF) through lumbar puncture for cytological examination and genetic testing to further evaluate brain involvement.

While no heterotypic cells were detected in the CSF, the same *EGFR* 19Del mutation was detected in the CSF ctDNA (Figure 1). In both the primary tumor and CSF biopsies, the *EGFR* mutation was detected at a relatively high mutant allele frequency (MAF, 53.22% and 35.87%, respectively) (Table 1). Notably, *RARA* c.1165G>A and *HGF* c.545G>A were only present in the CSF, which might be due to inter-tumor heterogeneity between the primary and metastatic lesions

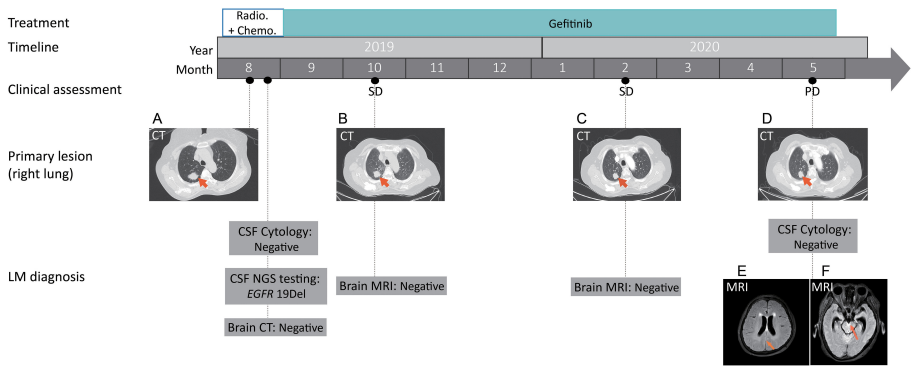


FIGURE 1
Treatment timeline with representative clinical evaluation and radiologic images. Based on the pathological analysis of the right lung biopsy, together with results from CT scans of the chest, abdomen and radionuclide bone, the patient was diagnosed with a clinical stage IVb LADC, T1cN2M1c according to the eighth edition of The American Joint Committee on Cancer (AJCC) TNM classification.**(A)** Computed tomography (CT) scans showed the primary tumor mass (biopsy site, arrows) in the right lung at the time of diagnosis; **(B, C)** Chest CT showing stable disease (SD) following gefinitib treatment; **(D)** Chest CT showing stable disease in the primary lung site at disease progression. **(E, F)** Magnetic resonance imaging (MRI) showing the potential metastatic site (arrows) in leptomeninges. SD, stable disease; PD, progressive disease; NGS, next-generation sequencing. CSF, cerebrospinal fluid.

TABLE 1 Genetic alterations identified in the biopsies.

| Genes | Alternations | Nucleotide change | Mutant allele frequency | |
|---|---|---|---|---|
| | | | Tumor | CSF |
| *EGFR* | p.L747_P753delinsS | c.2240_2257dellins | 53.22% | 35.87% |
| *TP53* | p.R337L | c.1010G>T | 40.59% | 37.45% |
| *FLT4* | p.Y361H | c.1081T>C | 36.88% | 30.18% |
| *RELA* | p.P384T | c.1150C>A | 26.61% | 33.53% |
| *RPTOR* | RPTOR:exon8~BAIAP2:exon14 | – | 8.33% | 8.14% |
| *MED12* | Intron 25 splice site mutation | c.3577+1G>T | 5.97% | 8.57% |
| *ARAF* | p.Y500C | c.1499A>G | 1.24% | 14.01% |
| *RARA* | p.A389T | c.1165G>A | – | 22.77% |
| *HGF* | p.G182E | c.545G>A | – | 13.06% |

-, not detected; CSF, cerebrospinal fluid.

(Table 1). Despite potential central nervous system (CNS) involvement, the patient did not opt for the more CNS penetrant newer generation EGFR inhibitor, osimertinib, due to financial concerns. With gefitinib treatment, there was no significant change in the sizes of the primary lesion (Figures 1B, C) and bone and liver metastasis. Craniocerebral MRIs in October 2019 and February 2020 also showed no obvious abnormalities (Figure 1), which indicated stable disease (SD) according to the Response Evaluation Criteria in Solid Tumours (RECIST 1.1) Guideline. Thus, the patient continued on gefitinib treatment. In May 2020, while the primary lung tumor remained stable (Figure 1D), brain MRIs showed an abnormal linear signal shadow of the leptomeninges, and clinical evidence of severe headache, vomiting, and aggravated restlessness in the limbs, suggesting the possibility of LM (Figure 1E, F). Thus, at the time of disease progression, the patient had derived durable benefit from gefitinib for 9.17 months despite central nervous system involvement early on in the course of the disease, and the patient had only mild adverse reactions during gefitinib administration, including mild itching and fatigue.

## Discussion

Leptomeningeal metastasis is a severe complication in the late stage of malignant tumor, with poor prognosis and limited treatment options. Although positive CSF cytology remains the gold standard for the LM diagnosis, the sensitivity of initial is only 45-50% (3), which can be easily affected by the time and method of specimen collection and detection (6). On the other hand, neuroimaging methods, including CT and MRI, are also valuable in the investigation of LM. MRI demonstrates more sensitivity over CT (7) with a sensitivity of 65% (8). Nevertheless, it has been reported that 20-30% of patients with confirmed LM had a false-negative MRI (3, 4). An accurate diagnosis of LM would rely on both CSF cytology and MRI evaluations, along with clinical manifestations. Regardless, given their low sensitivity, developing other diagnostic approaches is urgently needed.

NGS has emerged as a novel approach for the diagnosis of LM. Recent studies have shown that ctDNA in CSF can be used to characterize and monitor LM. In patients with LM in the BLOOM study, *EGFR*-mutated ctDNA was identified in CSF, the level of which decreased during treatment in correlation with improved neurological function or MRI result (5, 9). Besides, ctDNA analysis can reveal potentially druggable mutations that inform clinical decisions. In our study, the oncogenic mutations identified in the CSF ctDNA were highly concordant with that found in the primary tumor tissue. Although the brain lesion was the eventual cause of progressive disease in this case, the patient remained stable on gefitinib treatment for as long as 9.17 months. We speculate that the patient could have derived more intracranial benefit from drugs such as osimertinib, which was designed for a better CNS penetration to target LM (10).

Furthermore, our case has shown that CSF ctDNA may enable a more sensitive LM diagnosis, which was positive much earlier than any of the CSF cytology or radiographic evidence of LM involvement. In particular, no LM was seen at baseline brain CT, CSF cytology, or the subsequent series of brain MRI tests until the patient experienced PD in May 2020. By contrast, the NGS test of the CSF ctDNA was positive and revealed an oncogenic driver that was consistent with the primary lung tumor tissue from the initial diagnosis. Meanwhile, the patient had been experiencing symptoms such as headache, nausea, aphasia, restless limbs, and a sudden vision loss in both eyes throughout the treatment, which further supports the false negativity of CSF cytology or the radiologic exams. Although we could not confirm whether the patient had LM at the time of disease diagnosis, the patient's persistent brain abnormality-related symptoms and oncogenic mutations in the CSF strongly support the early involvement of LM. Therefore, our result demonstrates that NGS tests might be able to capture early LM signals in this patient with higher sensitivity than any of the traditional methods. Unfortunately, in this single case, we could

not find more direct clues of early LM besides ctDNA results and clinical evidence. Future large-scale studies should be conducted to further verify these findings. Nevertheless, our case suggests combinatorial testing of ctDNA, CSF cytology and MRI might be the trend in medical diagnosis for early detection of disease progression in patients with potential LM involvement.

In general, early diagnosis of LM involvement with sensitive diagnostic methods would be highly valuable in patients with more localized disease at presentation as it provides the patients with more potential surgical and therapeutic options, such as the addition of local brain radiotherapy (LBRT) and the choice of drugs with a better CNS penetration like osimertinib. In particular, for LADC patients with a low level of disease burden, we believe LBRT is a promising approach for controlling brain tumor growth. In our case, although the patient presented with highly metastatic disease at the time of diagnosis, he still could have derived more intracranial benefit, and a longer PFS from osimertinib rather than gefitinib as the eventual cause of progression was an increased size of the brain lesion, if not for financial concerns.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Shandong Cancer Hospital and conformed to the provisions of the Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from the patient's family member for the publication of this case report and any accompanying images.

## Author contributions

SHY conceived and supervised the study. YJB collected clinical data from patients and wrote the paper. QXY, NL and XLL assisted with the clinical data collection and analysis. DW and JWL revised the manuscript and designed the table and image. All authors contributed to the article and approved the submitted version.

## Funding

This work was supported by the Natural Science Foundation of China (NSFC81872475, NSFC82073345).

## Acknowledgments

We would like to thank the patient and his family for giving consent for publication.

## Conflict of interest

Authors JL and DW were employed by Nanjing Geneseeq Technology Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 26 January 2023
DOI 10.3389/fonc.2022.1055968



OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology

RECEIVED 28 September 2022
ACCEPTED 30 November 2022
PUBLISHED 26 January 2023

CITATION
Chan HT, Nagayama S, Otaki M, Chin YM, Fukunaga Y, Ueno M, Nakamura Y and Low S-K (2023) Tumor-informed or tumor-agnostic circulating tumor DNA as a biomarker for risk of recurrence in resected colorectal cancer patients. *Front. Oncol.* 12:1055968. doi: 10.3389/fonc.2022.1055968



# Tumor-informed or tumor-agnostic circulating tumor DNA as a biomarker for risk of recurrence in resected colorectal cancer patients

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**Introduction:** Circulating tumor DNA (ctDNA) has been increasingly recognized as a promising minimally-invasive biomarker that could identify patients with minimal residual disease and a high risk of recurrence after definitive treatment. In this study, we've compared the clinical utility and sensitivity of 2 different approaches to ctDNA analyses: tumor-informed and tumor-agnostic in the management of colorectal (CRC) patients. The clinical benefits of a single timepoint ctDNA analysis compared to serial ctDNA monitoring after definitive treatment were also evaluated to uncover the ideal surveillance protocol.

**Methods:** Patient-paired resected tumor tissues, peripheral blood cells, and a total of 127 pre-operative and serial plasma cell-free DNA (cfDNA) samples after definitive treatment from 38 CRC patients that had undergone curative intent surgery were analyzed using a commercial NGS cfDNA panel.

**Results:** Up to 84% (32/38) of the recruited patients were detected with at least 1 genomic alteration from the tumor tissues that could be monitored using the tumor-informed ctDNA approach and none of the detected alterations were clonal hematopoiesis (CH) related. In contrast, 37% (14/38) of patients were detected with at least 1 monitoring alteration after exclusion of CH mutations using the tumor-agnostic approach. Serial plasma samples after definitive therapy were available for 31 patients. In the landmark ctDNA analysis, 24% (7/29) of patients had detectable ctDNA and were more likely to relapse than ctDNA-negative patients ($p < 0.05$). The landmark analysis sensitivity and specificity for recurrence were 67% and 87%, respectively. The incorporation

of longitudinal ctDNA analysis at 6-months intervals improved the sensitivity to 100%. The median variant allele frequency (VAF) of the ctDNA mutations detected during surveillance was 0.028% (range: 0.018-0.783), where up to 80% (8/10) of the mutations were detected at VAF lower than the tumor-agnostic detection limit of 0.1%. Utilizing the tumor-agnostic approach reduced the recurrence detection sensitivity to 67% (4/6). Serial ctDNA analyses predicted disease recurrence at a median of 5 months ahead of radiological imaging.

**Conclusion:** Longitudinal monitoring using tumor-informed ctDNA testing shows high analytical sensitivity, low probability of false-positive results due to CH mutations, and improved sensitivity in detecting recurrence which may modify the clinical management of CRC.

KEYWORDS

**circulating cell-free DNA, liquid biopsy, minimal residual disease, colorectal cancer, recurrence risk**

## Introduction

Colorectal cancer (CRC) is the third most common cancer in Japan leading to an estimated 51,000 deaths in 2019 (1). Despite improved surgical procedures and advances in treatment regimens, 30-40% of CRC patients develop recurrence within 5 years of post-definitive treatment (2, 3). The current standard of care for localized disease is surgery with complete mesocolic/mesorectal excision followed by adjuvant chemotherapy (ACT) in selected patients (4). Surgery-alone is currently recommended for all stage I patients with a 5-year-survival rate of over 90% (5). In contrast, ACT is recommended for high-risk stage II patients which are defined as those with poor prognostic features (T4 tumors, perforated tumors, bowel obstruction, perineural or lymphovascular invasion, poorly or undifferentiated tumor grade, grade BD3 tumor budding, and <12 lymph nodes removed) (2, 5, 6). However, the magnitude of survival benefits of adding ACT for high-risk stage II remains unclear and controversial, where a considerable number of patients have to suffer from the adverse effects of ACT without significant clinical benefit (7–9). For stage III CRC patients, up to 50% of patients can be cured from surgery alone and 20% of patients benefit from the additional ACT (10). Despite this, ACT is currently recommended for all stage III patients. Furthermore, up to 30% of the ACT-treated stage III patients would still develop recurrence, suggesting the need for additional therapy in this subset of patients (11). Effective clinical tools or biomarkers that identify patients who are more likely to recur after curative-intent therapy and may benefit from systemic treatments are greatly needed.

Circulating tumor DNA (ctDNA) has been increasingly recognized as a promising minimally-invasive biomarker that could detect minimal residual disease (MRD) in blood samples after definitive treatment and identify patients with a higher risk of recurrence (12–21). Several prospective interventional clinical trials are also underway to evaluate the clinical benefits of utilizing ctDNA for ACT guidance and detection of recurrence during disease surveillance (22–27). The majority of the conducted studies and clinical trials were designed based on tumor-informed ctDNA assays (12, 15–18, 20). A tumor-informed assay relies on initial genomic profiling of the tumor tissues to identify tumor-derived alterations that could be evaluated and monitored using ctDNA. This approach has shown high analytical sensitivity with an improved risk of recurrence prediction (28). However, recent studies have shown that tumor-agnostic assays, that are independent on prior tumor genomic knowledge of the patient, may also achieve comparable sensitivity to tumor-informed assay in identifying patients with a higher risk of recurrence (19, 21). Given the independence of tumor tissue sequencing, tumor-agnostic assays may offer a more rapid turnaround time with reduced cost. Nevertheless, limited studies have directly compared the clinical feasibility and sensitivity of both approaches.

The ongoing clinical trials have primarily focused on evaluating the clinical benefits of a single-time point ctDNA analysis (landmark ctDNA analysis) after definitive therapy for treatment guidance in CRC patients (22, 23). Based on the results from the current studies, it is evident that patients with detectable ctDNA at the landmark timepoint show a significantly inferior recurrence-free survival (RFS) compared to ctDNA-negative patients (12, 13, 17, 20). However, the results of these studies also indicated that 10-25% of the patients lacking detectable landmark ctDNA also recurred (12, 17, 19–21). These findings highlighted the potential inadequacy of a single timepoint ctDNA analysis to predict recurrence and guide treatment decisions. The integration of longitudinal and surveillance ctDNA analysis may improve the prediction of

recurrence risk (16, 19, 20), however, the most optimal approach and surveillance protocol for identifying high-risk CRC patients remain unclear.

In this study, we report findings from a prospective and observational study that compared the clinical feasibility and sensitivity of utilizing a commercially available cfDNA panel with a tumor-informed, and tumor-agnostic approach to predict the risk of recurrence in the same resected CRC patient cohort. Plasma ctDNA analysis was performed before surgical resection and during routine follow-up after curative-intent treatment to assess the clinical utility of both landmark and longitudinal ctDNA monitoring in predicting the risk of recurrence.

# Materials and methods

## Patient cohort and sample collection

A total of 38 patients with histologically confirmed colorectal adenocarcinoma from the Cancer Institute Hospital of Japanese Foundation for Cancer Research in 2018 were included in this study. All eligible patients included in this study were pathologically confirmed as stage I to IV colorectal adenocarcinoma and were not subjected to chemotherapy or radiation therapy before tumor resection. The clinical and pathological information was obtained from the pathology reports and the electronic medical record for each patient. This study was approved by the ethical committee in Japanese Foundation for Cancer Research (IRB-2013-1093). The study design and details of blood collection time points are shown in Figure 1A. Tumor tissues and peripheral blood samples were collected at the time of surgery. Blood samples were collected longitudinally after surgical resection and completion of adjuvant chemotherapy and evaluated retrospectively. For patients who only underwent surgical resection, monitoring blood samples were collected at 6 months, 12 months, and 24 months after surgery. For patients administered ACT, blood samples were collected at 0, 6, 12, and 18 months after completion of ACT. Surgically-resected tumor tissues were stored at -80 ˚C until DNA extraction. The collection and processing of blood have been described previously (29–32). Briefly, 14 mL of peripheral blood was collected using EDTA-2Na tubes (Terumo, Tokyo, Japan) and were centrifuged at 2,000x *g* at 4 ˚C for 10 minutes within 30 minutes after the collection. The obtained plasma samples were further centrifuged at 16,000x *g* at 4 ˚C for 10 minutes to remove cell debris. The separated plasma and peripheral blood cells (PBCs) were stored at -80 ˚C until nucleic acid extraction.

## DNA/RNA extraction

A total of 127 pre-and post-operative plasma samples were collected from 38 patients and the cell-free total nucleic acid (cfTNA), which includes both DNA and RNA, was extracted using the MagMAX Cell-Free Total Nucleic Acid Isolation kit (Applied Biosystems) according to the manufacturer's protocol. Genomic DNA was extracted from frozen tumor tissues using the Allprep DNA Mini Kit (Qiagen) according to the manufacturer's protocol. Frozen PBCs samples were treated with the Red Blood Cell Lysis buffer following the manufacturer's protocol (BioLegend). The treated PBCs were counted using the Invitrogen Countess Automated Cell counter (Fisher Scientific) and DNA from a total of $2 \times 10^6$ PBCs was extracted using the Allprep DNA Mini Kit. Extracted cfTNA and genomic DNA (both PBCs and tumor tissues) were quantified using Qubit DNA HS Assay Kit and Qubit DNA Broad range assay kit (Life Technologies), respectively. The quality of the extracted DNA was assessed using the TapeStation system (Agilent) either *via* Genomic DNA ScreenTape (tumor and PBCs DNA) or High Sensitivity D5000 ScreenTape (cfTNA) (Agilent).

## Library preparation and targeted next-generation sequencing

Targeted NGS for cfTNA was carried out using the Oncomine Pan-Cancer Cell-Free Assay following the manufacturer's protocol (Life Technologies), with an input of 8.3-20 ng of cfTNA. Oncomine Pan-Cancer Cell-Free Assay is an amplicon-based ctDNA targeted assay with unique molecular identifiers (UMIs) and detects single nucleotide variants (SNVs), copy number variations (CNVs), and gene arrangements across 52 genes. Library construction was undertaken as previously described (29–32). Libraries were multiplexed for templating on the Ion Chef Instrument and subsequently sequenced on the Ion S5 Prime System using the Ion 540 or 550 Chip Kit. Both tumor and PBCs DNA were mechanically sheared to 150 bps before library construction. A similar sequencing methodology was applied for DNA extracted from tumor tissue and PBCs with an input of 20 ng.

## Sequencing data analysis and statistical analysis

Sequencing alignment, quality control analysis, and variant calling were conducted by the Torrent Suite Software version 5.10.1 (Thermo Fisher Scientific) and Ion Reporter version 5.10 (Thermo Fisher Scientific). In brief, raw sequence files were aligned to hg19 using the Torrent Mapping Alignment Program (TMAP) with default analysis parameters. The subsequent BAM files generated were then further analyzed by Oncomine TagSeq Pan-Cancer Liquid Biopsy w2.1 version 5.10 with the following modifications for a positive variant calling: (i) A minimum of 3 reads with the same UMI were required to form a functional family. (ii) Under tumor-agnostic calling, a minimum of 3 variant supporting functional families with a minimum variant allele

frequency (VAF) of 0.1% were required to make single nucleotide variants (SNVs), multi nucleotide variants (MNVs), and insertions/deletions (INDELs) callings for a known cancer hotspot mutation. (iii) Under the matched tumor-informed manner where the mutation was previously detected from the tumor tissue of a patient, a minimum of 1 variant supporting functional family was required to make SNVs, MNVs, and INDELs callings. Variants were annotated using Oncomine Pan-Cancer Annotation version 1, a proprietary list of databases. RFS was assessed by standard radiologic criteria. RFS was measured from the day of completion of definitive treatment to the first verified radiological recurrence. For patients whose treatment was only surgery, RFS was measured from the day of surgical resection. For patients who received adjuvant chemotherapy, RFS was measured from the day of completion of chemotherapy. The definition of RFS was similarly described in a previous study (19). Patients were censored at the date of the last follow-up. Survival analysis was performed using the Kaplan-Meier method. Cox proportional hazards regression analysis was used to assess the association of ctDNA with RFS. Differences in pre-operative ctDNA detection rate between tumor-informed and tumor-agnostic approaches and differences in recurrence rate between ctDNA positive and ctDNA negative groups were assessed using Fisher's exact test. All p-values were based on two-sided testing and differences were considered significant at $p<0.05$. Statistical analysis was performed using R Statistical software (Version 4.0.5).

# Results

## Patient characteristics

An overview of the study workflow is presented in Figure 1B. A total of 38 CRC patients were included in this study. The clinical and pathological characteristics of the patients are shown in Table 1 and Table S1. The median age of the patients at the initial sample collection was 66 years old and 63% of the patients were male. Among them, 53% (20/38) were diagnosed with stage I or II, and 47% (18/38) were diagnosed with stage III or IV. All patients underwent surgical resection with curative intent. One of the three Stage IV patients received simultaneous resection of the primary tumor and solitary liver metastatic lesion, and the remaining two Stage IV patients underwent the resection of the solitary peritoneal dissemination along with the primary tumor. Six patients with no genomic alterations detected from tumor tissues and one patient who developed clinical recurrence before the collection of the first post-operative blood sample were excluded for long-term follow-up (Figure 1B).

ACT was administered to 18/31 longitudinally monitored patients with a median chemotherapy duration of 179 days (Table S2). Radiological recurrence was detected in 19% (6/31) of the evaluated patients with a median time to recurrence of 8.5 months after definitive treatment. The median follow-up time was 20 months (14-27 months) after definitive treatment for recurrence-free patients.

## Detection of pre-operative ctDNA using the tumor-agnostic or tumor-informed approach

Cell-free TNA was successfully extracted from all 127 plasma samples with an average concentration of 8.0 ng per mL of plasma (1.6-36.9 ng/mL; Table S3). All cfTNA samples were successfully sequenced with an average cell-free DNA (cfDNA) input of 17.5ng to a median raw coverage of 54,772x and a median collapsed coverage of 4,296 (Table S3). The average library conversion rate was 82% (Table S4). Under a tumor-agnostic setting with a VAF detection limit of 0.1%, a total of 27 SNVs and Indels were detected in the pre-operative plasma samples from 50% (19/38) of the patients with a median VAF of 0.37% (Range: 0.10-14.25%; Table S5). Genomic DNA extracted from pre-operative PBCs was sequenced to a comparable coverage to the plasma cfDNA (raw coverage of 48,622x and median collapsed coverage of 2,581x; Table S6) to identify the possible clonal-hematopoiesis (CH) mutations. A total of 11 mutations were detected from PBCs and 9 of them were simultaneously detected from plasma cfDNA (Table S7). CH mutations constituted up to 33.3% (9/27) of the total mutations detected from plasma cfDNA (Table S5). After the exclusion of CH mutations, 37% (14/38) of the evaluated patients harbor at least one tumor-derived mutation from plasma cfDNA for longitudinal monitoring.

The genomic profile of tumor tissues was evaluated to compare the detection of pre-operative ctDNA between tumor-agnostic and tumor-informed approaches. Genomic DNA isolated from tumor tissues was sequenced to a median coverage of 18,450x (Table S8). A total of 61 somatic genomic alterations in 10 genes were identified using a VAF cut-off of 1% (Table S9). The mutational landscape of the detected mutations is summarized in Figure 2, where the most commonly mutated genes were *TP53* (41%), *KRAS* (21%), *PIK3CA* (11%), and *APC* (10%) (Supplementary Figure 1). Using a VAF cut-off of 1%, none of the mutations detected from tumor tissues were present in the PBCs (Table S10). In contrast to the tumor-agnostic approach, up to 84% (32/38) of the patients harbor at least one mutation from the tumor tissue for subsequent plasma cfDNA monitoring (Figure 3A). Of those mutation calls from tumor tissues, 29.5% (18/61) were concordantly detected from pre-operative plasma without the aid of prior patient-specific tumor genomic knowledge (Figure 3B). An additional 24.6% (15/61) of the alterations from tumor tissues were detected from plasma cfDNA using the tumor-informed approach, with a minimum of 1 variant supporting functional family (Method) and a median observed VAF of 0.04% (Range: 0.02-0.09% Figure 3B). The pre-operative ctDNA detection rate was significantly higher in stage

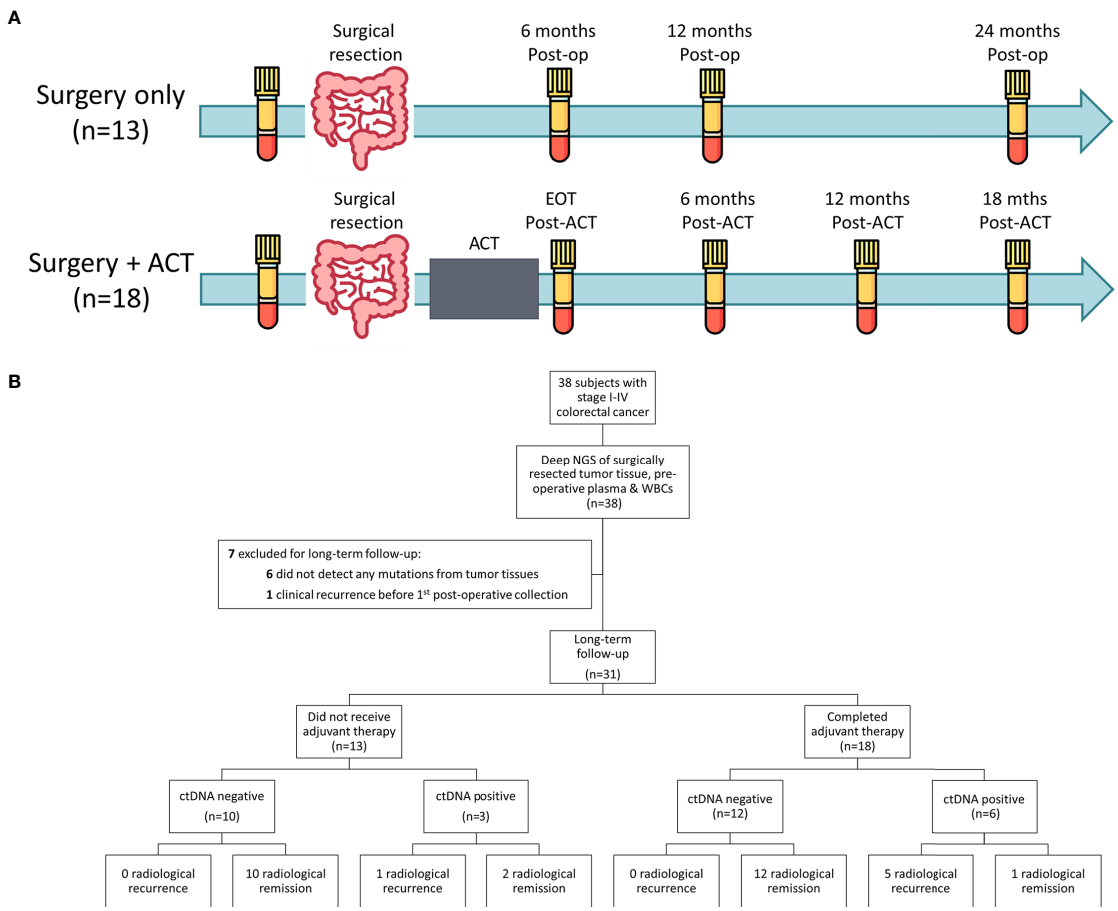


FIGURE 1
Study design and patient enrolment. **(A)** Study design and overview of the blood collection time points. For patients treated with surgery alone (n=13), blood samples were collected before surgical resection and at 6 months, 12 months and 24 months after surgery (Post-op) or until radiological recurrence. For patients who received adjuvant chemotherapy (ACT), blood samples were collect prior to surgical resection, at the end of ACT (EOT) and at 6 months, 12 months and 18 months after completion of ACT (Post-ACT) or until radiological recurrence. **(B)** A total of 38 stage I-IV CRC patients that undergone surgical resection with curative-intent were included in this study. After exclusion, 31 patients were included for long-term follow-up analysis. Circulating tumor DNA status was determined based on the longitudinal tumor-informed ctDNA analysis after definitive treatment.

I-III CRC patients using the tumor-informed approach compared to the tumor-agnostic approach with a detection rate of 66% and 31% respectively (p-value = 0.008; Figure 3C and Supplementary Figure 2). In contrast, ctDNA was detected in all three stage IV patients using both approaches. Due to the higher detection sensitivity observed using the tumor-informed approach, all subsequent post-therapy cfDNA samples were analyzed using the tumor-informed approach.

## Landmark ctDNA analysis after definitive treatment and risk of recurrence

'Landmark' ctDNA analysis was defined as the detection of ctDNA from the first plasma sample drawn after the completion of definitive treatment (surgery alone or completion of adjuvant chemotherapy). For patients who were subjected to surgery alone, the landmark sample was taken approximately 5 months after surgery (median: 162 days, Figure 4A). For patients who have received adjuvant chemotherapy, the first plasma sample was taken approximately 1 month after completion of adjuvant chemotherapy (median 22.5 days, Figure 4B). Landmark plasma samples were available for 29 of the 31 patients with long-term follow-up, and ctDNA was detected in 24% (7/29) of samples (Figure 5A). The recurrence rate was significantly higher for ctDNA-positive patients at 57% (4/7), compared to 9% (2/22) for negative patients ($p<0.05$, Figure 5A). Sensitivity and specificity for detection of recurrence were 67% and 87% respectively (Figure 5A). Recurrence-free survival for patients with detectable landmark ctDNA was

TABLE 1 Clinical and pathological characteristics of the study cohort.

| Clinico-pathologic features | No., (%) (n=38) |
|---|---|
| Age, years | |
| Median | 66 |
| Range | 42-88 |
| Gender | |
| Male | 24 (63) |
| Female | 14 (37) |
| Stage | |
| I | 7 (18) |
| II | 13 (34) |
| III | 15 (40) |
| IV | 3 (8) |
| Tumor Site | |
| Cecum | 6 (16) |
| Ascending | 4 (11) |
| Transverse | 4 (11) |
| Descending | 2 (5) |
| Sigmoid | 5 (13) |
| Rectum | 17 (45) |
| Differentiation | |
| Well | 8 (21) |
| Moderate | 28 (74) |
| Poor | 2 (5) |
| T Stage | |
| T1 | 4 (11) |
| T2 | 4 (11) |
| T3 | 20 (53) |
| T4 | 10 (26) |
| Nodal involvement | |
| N0 | 21 (55) |
| N1,N2,N3 | 17 (45) |
| Tumor size (mm) | |
| Median | 40 |
| Range | 12-90 |
| Lymphatic Invasion | |
| No | 20 (53) |
| Yes | 18 (47) |
| Venous Invasion | |

*(Continued)*

TABLE 1 Continued

| Clinico-pathologic features | No., (%) (n=38) |
|---|---|
| No | 9 (24) |
| Yes | 29 (76) |
| Baseline CEA elevated (>5 ng/mL) | |
| No | 26 (68) |
| Yes | 12 (32) |
| Baseline CA 19-9 elevated, (>37 U/mL) | |
| No | 35 (92) |
| Yes | 3 (9) |
| Baseline CA-125 elevated,(>46 U/mL) | |
| No | 38 (100) |
| Yes | 0 (0) |

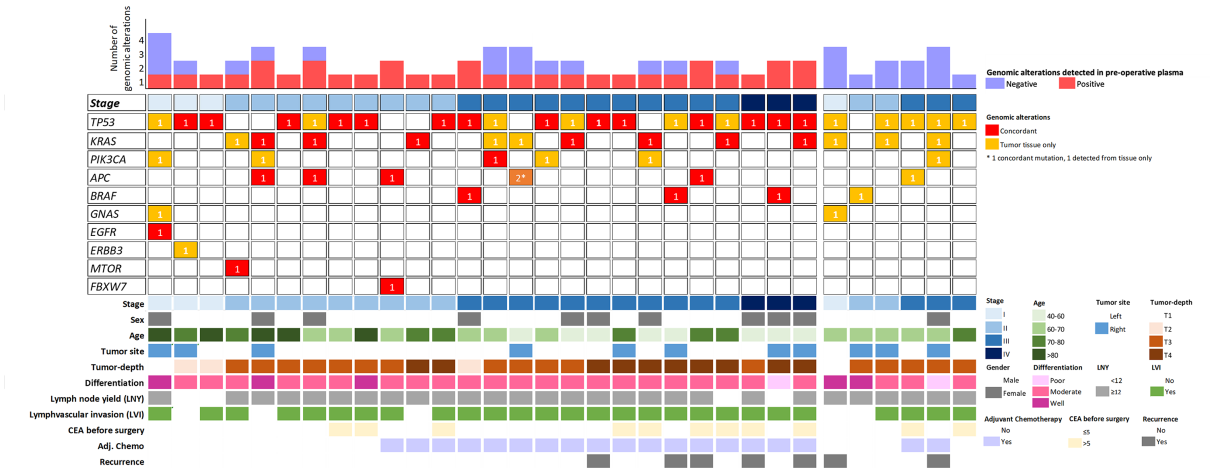


FIGURE 2
A schematic representation of the genomic alteration distribution detected from tumor tissues and pre-operative plasma cfDNA. Each column represents a sample, and it is classified according to the pathological stage. Six patients with no detectable ctDNA were group on the right and six patients with no genomic alterations detected from the tumor tissues were excluded from the plot.

significantly shorter than those with negative ctDNA and a 12.4 times higher risk of developing recurrence (Figure 5B, HR: 12.4, P<0.001).

## Longitudinal ctDNA and risk of recurrence

To investigate whether longitudinal ctDNA analyses could improve the sensitivity for recurrence prediction compared to landmark analysis, subsequent plasma samples were evaluated for all 31 patients with long-term follow-up. A total of 3 or 4 serial plasma samples after the end of definitive treatment (surgery only or ACT, respectively) from each patient were drawn for the longitudinal ctDNA analysis (Table S10). Detection of ctDNA at any serial plasma samples until the development of clinical recurrence would be considered ctDNA-positive. Overall, 60% (6/10) of patients who were tested ctDNA-positive during surveillance developed radiological recurrence, whereas none of the 21 patients that remained ctDNA-negative throughout the surveillance developed clinical recurrence, giving a negative predictive value of 100% (Figure 6A, p<0.001). The incorporation of serial ctDNA

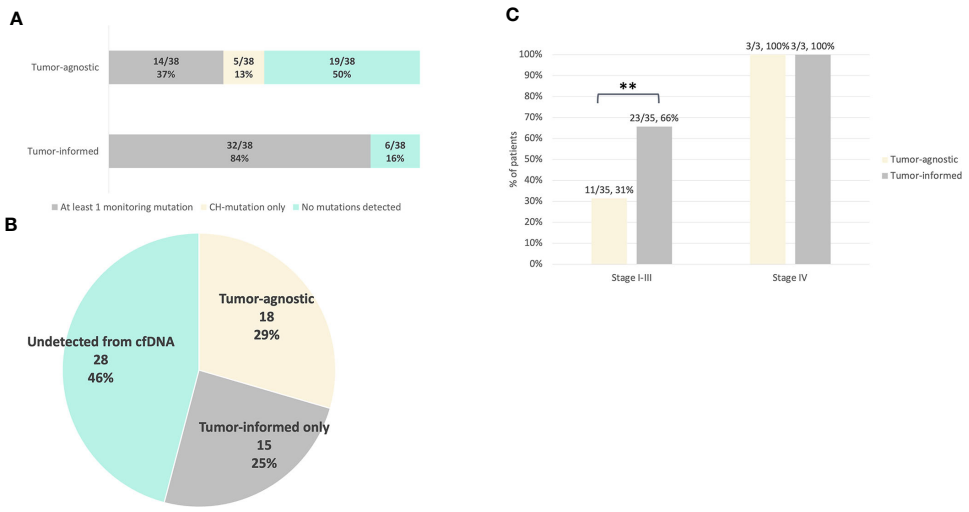


FIGURE 3
Comparison of pre-operative ctDNA detection between the tumor-informed and tumor-agnostic approach. For tumor-informed approach, mutation detection was evaluated based on the genomic profile of the tumor tissues. For tumor-agnostic evaluation, mutation detection was evaluated based on alterations detected from pre-operative plasma cfDNA with VAF ≥ 0.1% without previous knowledge of the tumor genomic profile (Methods). Clonal hematopoiesis (CH) mutations were excluded from both approaches. **(A)** Number of patients detected with at least one monitoring tumor-derived mutation for ctDNA surveillance using the tumor-agnostic or tumor-informed approach. **(B)** Proportion of tumor mutations that were detected from pre-operative plasma cfDNA using the tumor-agnostic or tumor-informed approach. A total of 61 mutations were detected from tumor tissues, 18/61 were detected from plasma cfDNA using the tumor-agnostic approach and additional 15 alterations were detected from pre-operative plasma cfDNA using the tumor-informed assay. **(C)** Pre-operative ctDNA detection rates in stage I-III (n=35) and stage IV (n=3) patients using tumor-informed and tumor-agnostic ctDNA testing (p value = 0.00806). The p value was obtained from two-by-two Fisher's exact tests. ** $p < 0.01$.

increased the sensitivity of prediction for recurrence from 67% to 100% (Figure 6A), and ctDNA-positive patients remained to have a significantly lower RFS compared to negative patients (HR:19.3, p<0.0001, Figure 6B). The median VAF of the detected mutations was 0.028% (range: 0.018-0.783), and up to 80% (8/10) of the mutations were detected at VAF lower than the tumor-agnostic detection limit of 0.1% (Supplementary Figure 3A). Consequently, only 67% (4/6) of the recurrence cases could be detected using tumor-agnostic ctDNA monitoring (Supplementary Figures 3B, C).

Longitudinal ctDNA analysis detected recurrence earlier than radiological imaging in 4 of the 6 recurrence patients (Supplementary Figure 4). The median time for disease

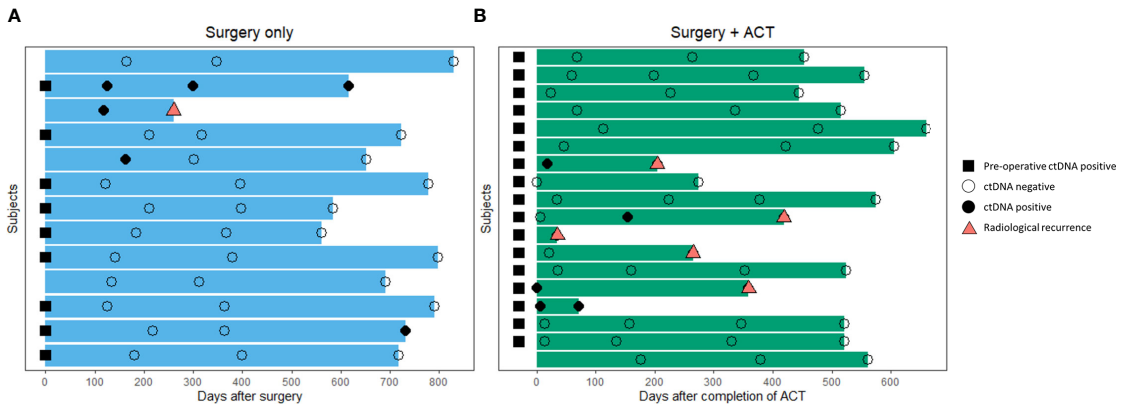


FIGURE 4
Overview of blood samples analyzed for landmark and longitudinal ctDNA testing after definitive therapy. **(A)** Patients that received curative-intent surgery alone (n=13) **(B)** Patients that received curative-intent surgery followed by adjuvant chemotherapy (n=18).

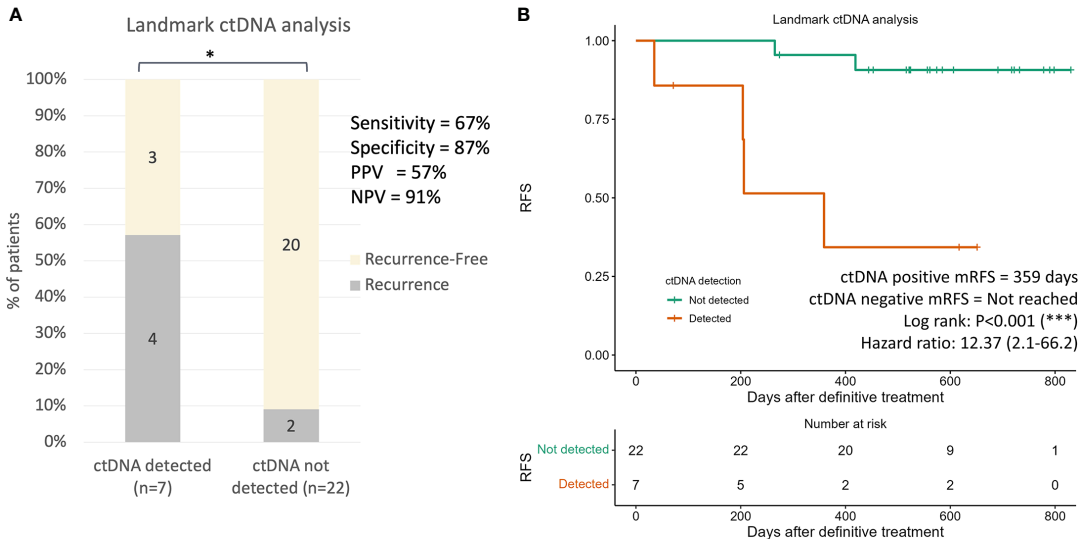


FIGURE 5
Landmark ctDNA analysis for recurrence risk assessment. **(A)** Recurrence rates in patients with detected ctDNA and undetected ctDNA at landmark ctDNA analysis (*p* value=0.01080). The *p* value was obtained from two-by-two Fisher's exact tests. **(B)** Kaplan-Meier plot of recurrence-free survival stratified for ctDNA detection in landmark ctDNA analysis. * p < 0.001; *** p < 0.0001

recurrence determined by ctDNA analyses was 77 days after definitive treatment, compared to 236 days determined by radiological imaging, resulting in a ctDNA median lead time of 159 days (Figure 7, p<0.05). In the 3 recurrence cases where plasma samples were available at the time of radiological detection of relapse, ctDNA remained positive with an increase in the VAF of the detected mutations in all patients at the time of radiological recurrence (mean 10.1 folds, range: 2.9-

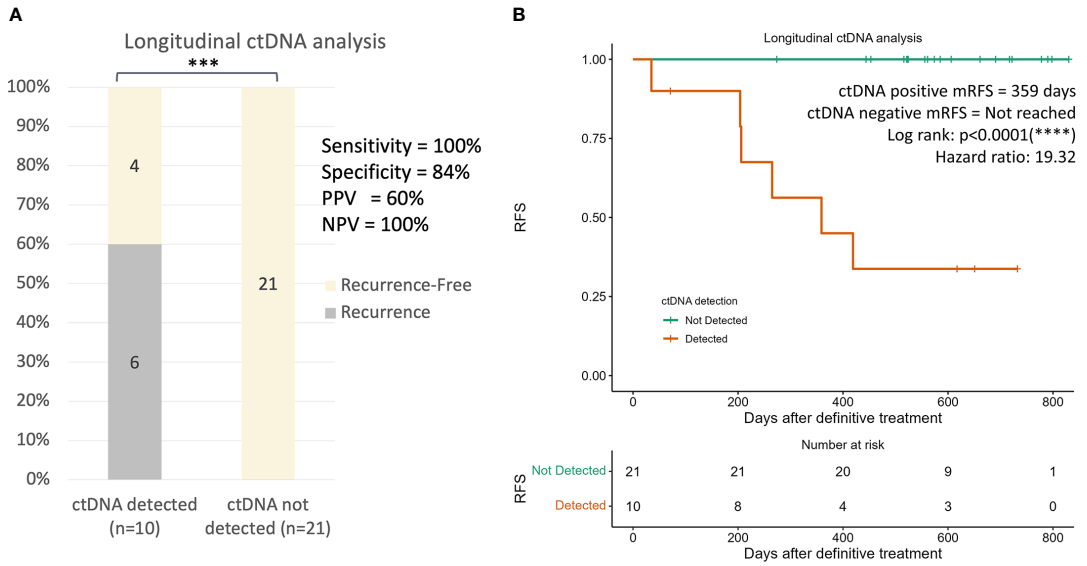


FIGURE 6
Longitudinal ctDNA analysis for recurrence risk assessment. **(A)** Recurrence rates in patients with detected ctDNA and undetected ctDNA at longitudinal ctDNA analysis (*p* value=0.002852). Detection of ctDNA at any serial plasma samples until the development of clinical recurrence would be considered as ctDNA-positive. The *p* value was obtained from two-by-two Fisher's exact tests. **(B)** Kaplan-Meier plot of recurrence-free survival stratified for ctDNA detection in longitudinal ctDNA analysis. ***p < 0.05; ****p < 0.001.

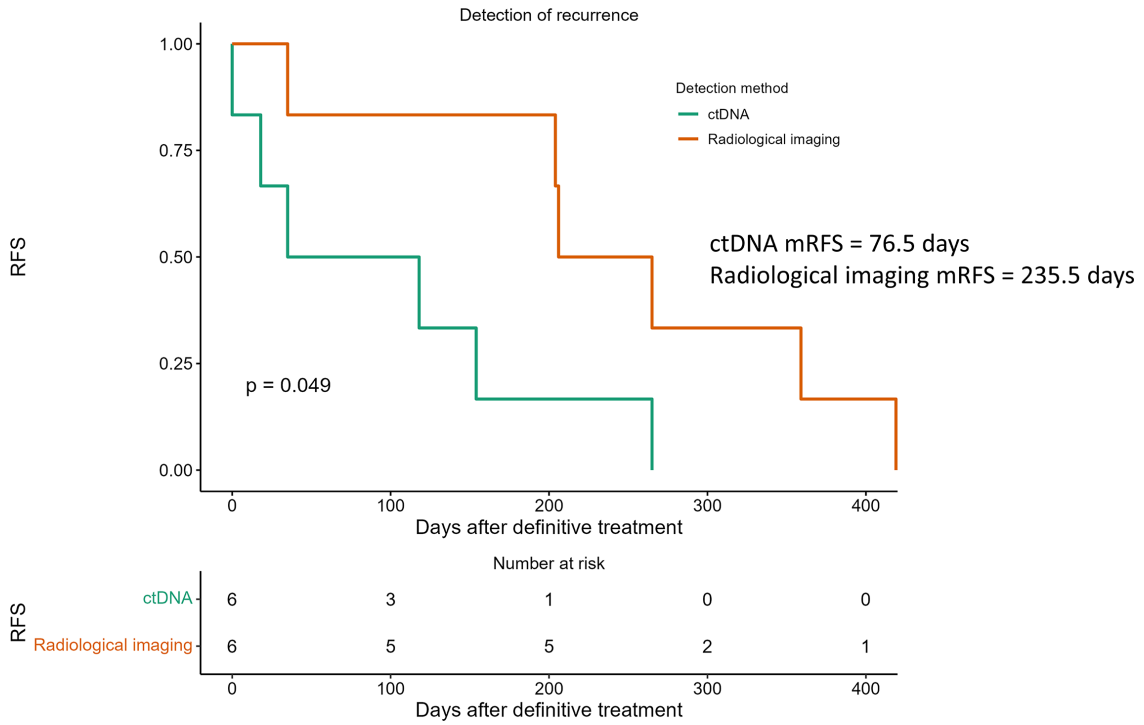


FIGURE 7
Detection of recurrence using longitudinal ctDNA analysis and standard radiological imaging. ctDNA (green line) analyzed at serial time points after definitive treatment predicted recurrence with a median lead time of 159 days over radiological recurrences (red line). P value determined by log-rank test.

16.8 folds), indicating the markedly increase in tumor burden while the patients awaited radiologic detection of the recurrence (Supplementary Figure 5).

## Discussion

A sensitive prognostic biomarker that accurately identifies CRC patients with a higher risk of recurrence after curative-intent therapy could potentially improve their survival outcome. The results from our observational study are in concordance with previously published studies where ctDNA analysis after definitive treatment has demonstrated significant therapeutic promise in identifying patients with poor prognoses who may require further systemic treatments. Our study also emphasizes the importance of using a tumor-informed ctDNA assay with longitudinal surveillance to optimize the clinical utility of ctDNA in CRC management.

In the pre-operative context, we've observed that the tumor-informed ctDNA approach may offer superiority in detecting low tumor burden, especially in localized CRC patients over the tumor-agnostic approach. Up to 84% of our patient cohort was detected with at least one mutation from the tumor tissues that could be subsequently monitored using ctDNA, compared to only 37% of patients detected with at least one monitoring mutation using the tumor-agnostic approach. Without prior knowledge of the tumor genomic profile of the patient, the reason for the absence of ctDNA detection in the remaining 63% of patients would remain unknown as to whether it is due to the insufficient coverage of the targeted panel or low tumor fraction in the cfDNA, resulting further ctDNA monitoring for these patients to be clinically nonmeaningful. The significantly higher pre-operative ctDNA detection rate observed in stage I-III patients using a tumor-informed approach compared to the tumor-agnostic approach (66% and 31%, respectively) further illustrates the loss of sensitivity associated with the tumor-agnostic approach. The enhanced ctDNA detection sensitivity using a tumor-informed manner has been similarly demonstrated using the MSK-ACCESS ctDNA assay (33). The authors of the study reported that by performing variant calling in a matched tumor-informed manner, an additional 5% of variants were detected from the plasma cfDNA (33). Interestingly, ctDNA was detected in all three metastatic patients from our study cohort using both the tumor-informed and tumor-agnostic approach, highlighting that the impact of assay sensitivity is more prominent in patients with localized CRC. Furthermore, 33% of the alterations detected from pre-operative plasma cfDNA were of CH origin and the detection

frequency was consistent with previous studies (34–37). We've previously reported that the type and VAF of CH alterations detected from plasma cfDNA are often indifferent to ctDNA mutations, therefore patient-paired PBCs sequencing is essential to differentiate CH from tumor-derived alterations (29, 38). Misclassification of the origin of the alterations detected from cfDNA may lead to an erroneous interpretation of ctDNA analysis as an MRD (29). In contrast, using a VAF threshold of 1%, none of the patients in our study cohort were detected with CH alterations from the tumor tissues, precluding the need for additional sequencing of PBCs when adopting the tumor-informed approach.

In the post-definitive therapy context, 32% of our patient cohort was detected with ctDNA in at least one of the surveillance plasma samples yielding a recurrence detection sensitivity of 100%. Our observation aligns with previously reported tumor-informed ctDNA assays that produced sensitives ranging from 80-100% (15–18, 20). Up to 80% of the variants identified from the first ctDNA positive monitoring samples were detected at VAF below the tumor-agnostic detection limit (VAF<0.1%). Consequently, only 67% (4/6) of the recurrence cases could be detected using tumor-agnostic ctDNA monitoring. Similar detection sensitivity has been reported in a recent study that evaluated the feasibility of tumor-uninformed MRD detection using a plasma-only ctDNA assay with 73% of recurrence patients detected using ctDNA surveillance (19). Moreover, the authors also observed an increase in sensitivity by 18% with the incorporation of aberrant methylation patterns (19). Several other studies have also explored the use of epigenomic features in tumor-agnostic ctDNA assays for MRD detection. The reported sensitivity in detecting recurrence ranged from 63-90% (21, 39, 40). Current observations suggest genomic alterations-based tumor-agnostic ctDNA assays are unlikely to achieve comparable sensitivity as the tumor-informed approach in detecting and predicting recurrence in resectable CRC patients. The incorporation of other features is necessary for tumor-agnostic ctDNA assays to be used in clinical settings. Although the tumor-informed ctDNA approach outperforms the tumor-agnostic assay in terms of analytical and clinical sensitivity, the clinical utility of a tumor-informed ctDNA assay will inevitably be significantly reduced in cases where tumor tissues are not available or with limited tumor cellularity. This issue may be particularly relevant in patients that have undergone neoadjuvant therapy where resected specimens may have insufficient tissue or tumor content for genomic profiling due to following favorable treatment response.

Landmark ctDNA analysis after completion of definitive treatments is clinically attractive as it may facilitate immediate decision-making for initiation of adjuvant treatments or consolidation therapies. Consistent with previous studies, patients from our study cohort detected with positive ctDNA at the landmark sample showed an inferior RFS and 12 times higher risk of developing recurrence compared to ctDNA negative patients (12–21). Together, these results have suggested the possibility of treatment escalation in ctDNA-positive patients and treatment de-escalation in ctDNA-negative patients. The clinical benefits of the ctDNA-guided treatment approach in stage II CRC patients were recently reported for the first time from a phase II randomized prospective and interventional trial where the ctDNA-guided approach was able to reduce ACT usage in stage II patients without compromising RFS compared to the standard management (24). However, insufficient sensitivity of single timepoint analyses resulting in false-negative results may undermine the ctDNA-guided treatment regimen. Previous studies together with the aforementioned interventional trial have shown that close to 10% of patients with undetectable ctDNA after definitive treatment develop recurrence (12, 17, 24). This was similarly observed in our patient cohort where landmark ctDNA analysis was able to detect 67% of the recurrence cases with a relapse rate of 9% among the ctDNA negative patients. Future studies should explore the incorporation of ctDNA analysis with other circulating analytes such as circulating tumor cells, and non-genomic features to improve the sensitivity of landmark analysis in identifying patients with a higher risk of recurrence (41). One other strategy to alleviate the sensitivity-related issue is through longitudinal ctDNA testing. Previous studies have reported an increase in sensitivity from 40% to 88% using serial ctDNA analyses (17, 20). Similarly, we've also observed an increase in sensitivity from 67% to 100% through six-monthly ctDNA testing compared to a single timepoint analysis. These data highlight the importance of incorporating longitudinal ctDNA monitoring to maximize the clinical benefits of ctDNA analyses. Surveillance of ctDNA after definitive treatment showed a significant impact in recurrence detection compared to radiological imaging, demonstrating a lead time of 5 months that is similar to previously reported (12, 17, 20). In the cases where ctDNA analyses were also performed at the time of radiological recurrence, the ctDNA levels increased by a mean of 10 folds, indicating a marked increase of tumor burden during the 5 months of lead time. The early detection of residual disease from ctDNA analyses may allow earlier radiological imaging to be performed or timely adjustment of the treatment regimens. Some of the ongoing interventional trials have adopted the ctDNA surveillance approach in the study design where radiological imaging frequencies and ACT dosage regimen are modified according to the ctDNA status every 3 to 4 months (26, 27).

There are several limitations to our study. The small sample size and the low event rate limited our ability to compare the prognostic significance of ctDNA status with other known clinical features. Future studies with a larger cohort size are needed to validate this. The specificity of longitudinal ctDNA analysis observed in our cohort was lower than reported in previous observational studies (84% and 95%, respectively) (17, 20). One of the four ctDNA-positive patients was lost in follow-up, while one patient with detected ctDNA at the end of the

monitoring period was diagnosed with intrahepatic cholangiocarcinoma. Further evaluations for this patient are needed to confirm the origin of the radiologically detected tumor and to assess the discrepancies between ctDNA analysis and the clinical diagnosis. The remaining two patients were monitored for approximately 16 months after the first detection of ctDNA from the surveillance samples. In the study conducted by Henriksen et al., the authors observed 2 distinct tumor growth patterns where half of the recurrence patients showed slow growth with longer overall survival (20). It is unclear whether the slow growth pattern may account for the 2 ctDNA positive patients that remained undetected using radiological imaging by the end of the monitoring period. In this study, we've utilized a commercially available targeted cfDNA panel instead of establishing a personalized cfDNA assay based on the patient's tumor tissue genomic profile. Using a generic assay shortens the turnaround time and reduces the cost, however, up to 16% of the recruited patients from the study were excluded for further monitoring due to the lack of alterations detected from tumor tissues. The limited panel coverage for genes *APC* and *TP53*, and the use of a hot-spot-based variant calling bioinformatic pipeline with limited *de novo* calling may account for the reduced coverage observed. Improvements to the variant calling algorithm may overcome this drawback.

## Conclusion

In summary, we showed that ctDNA analysis with the tumor-informed approach outperforms the tumor-agnostic approach with higher analytical sensitivity, lower probability of false-positive results due to CH mutations, and improved sensitivity in detecting recurrence in resected CRC patients. Our results have also demonstrated that serial ctDNA monitoring after definitive treatment provides superior sensitivity over landmark ctDNA analyses in predicting and detecting recurrence. These data also suggest the clinical importance of incorporating longitudinal ctDNA monitoring to maximize the clinical benefits of liquid biopsy in CRC management.

## Data availability statement

The original contributions presented in the study are publicly available. This data has been uploaded to the NBDC database with the accession number JGAS000590.

## Ethics statement

The studies involving human participants were reviewed and approved by Ethical committee in Japanese Foundation for Cancer Research (IRB-2013-1093). The patients/participants provided their written informed consent to participate in this study.

## Author contributions

The study concept and design were conducted by SN, YN, and S-KL. Patient recruitment was carried out by SN, YF, and MU. Acquisition, analysis, or interpretation of data were performed by HC, SN, YC, and S-KL. Drafting of the manuscript was written by HC, SN, and S-KL. HC, SN, YC, YN, and S-KL contributed critical revision of the manuscript for important intellectual content. HC, MO provided technical support. SN, YF, MU, YN, and S-KL were the supervisors for this project. All authors have read and approved the final manuscript.

## Funding

This study was supported by the Council for Science, Technology and Innovation (CSTI), cross-ministerial Strategic Innovation Promotion Program (SIP), "Innovative AI Hospital System" (Funding Agency: National Institute of Biomedical Innovation, Health and Nutrition (NIBIOHN)).

## Acknowledgments

We gratefully acknowledge the patients and their families for participating in this study. We also thank Ms. Fumie Sakamoto, Ms. Rie Hayashi, Dr. Hideaki Matsuki, Ms. Saki Matsumoto and Dr. Hirofumi Aruga for their continuous technical support in this study.

## Conflict of interest

YC reported as an employee of Cancer Precision Medicine Inc., Japan. S-KL reported consulting or advisory roles with Cancer Precision Medicine Inc., Japan. YN reported consulting and advisory roles with OncoTherapy Science, Inc., Japan.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1055968/full#supplementary-material

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