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ORIGINAL ARTICLE

Non-invasive genotyping of metastatic colorectal cancer using circulating cell free DNA

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Abstract

Circulating cell-free DNA (ccfDNA) in plasma provides an easily accessible source of circulating tumor DNA (ctDNA) for detecting actionable genomic alterations that can be used to guide colorectal cancer (CRC) treatment and surveillance. The goal of this study was to test the feasibility of using a traditional amplicon-based next-generation sequencing (NGS) on Ion Torrent platform to detect low-frequency alleles in ctDNA and compare it with a digital NGS assay specifically designed to detect low-frequency variants (as low as 0.1%) to provide evidence for the standard care of CRC. The study cohort consisted of 48 CRC patients for whom matched samples of formalin-fixed, paraffin-embedded tumor tissue, plasma, and peripheral blood mononuclear cells were available. DNA samples from different sources were sequenced on different platforms using commercial protocols. Our results demonstrate that the ccfDNA sequencing with the traditional NGS can be reliably used in an integrated workflow to detect low-frequency somatic variants in CRC. We found a high degree of concordance between traditional NGS and digital NGS in profiling mutant alleles in ccfDNA. These findings suggest that the traditional NGS is a viable alternative to digital sequencing of ccfDNA at allele frequency above 1%. ccfDNA sequencing can not only provide real-time monitoring of CRC, but also lay the basis for its application as a clinical diagnostic test to guide personalized therapy.

Keywords Colorectal cancer, Low-frequency variant, ccfDNA, Next-generation sequencing.

Introduction

Both men and women have a lifetime risk of colorectal cancer (CRC) of about 1 in 20 [1,2]. It was estimated in 2017 that more than 125,000 people would be diagnosed with CRC, and more than 50,000 would die of the disease. Nearly 1 in 5 CRC patients have late-stage disease at diagnosis [3], which greatly diminishes treatment opportunities and contributes to a 5-year survival rate as low as 12% [4].

CRC accumulates gene mutations during its development; low-frequency variants are very common because of tumor heterogeneity; [5] accurately identifying low frequency variants is very important in the treatment and management of CRC.

Conventionally, the diagnosis of CRC is based on the findings of tumor biopsy analysis. However, solid tumor biopsies are highly invasive, costly, and carry all kinds of risks to patients [6,7]. Thus, a non-invasive method for diagnosing CRC and monitoring the disease in real-time is needed [8–10].

One potential alternative to solid tumor biopsy is using targeted Next Generation Sequencing (NGS) panels to genotype circulating cell-free DNA (ccfDNA) [9]. ccfDNA originates from both normal and tumor cells, and ccfDNA originating from

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tumor cells (referred to as ctDNA) carries the same genetic alterations as the primary and metastatic tumors [10,11]. Because it has the potential to yield information about all cell subpopulations and genetic alterations in the primary tumor and distant metastases [12], ccfDNA analysis could provide a better representation of a patient's CRC in real-time. In addition, the identification of low frequency variants present in ccfDNA enables one to better understand the temporal and spatial clonal evolution of tumors, tumor responses to therapy, and disease relapse. Previous studies have shown the existence of therapy-resistant clones or mutations as early as the therapy was initiated or after therapy begun (de novo acquisition) [13–16].

Generally, most targeted NGS technologies utilize either PCR amplification or hybridization capture-based strategies to enrich the target sequences during the preparation of sequencing-ready libraries [17]. Recently, in order to detect low frequency genetic variants (typically present in ccfDNA), unique molecular IDs have been incorporated into targeted NGS libraries to eliminate sequencing associated background errors, improve the specificity, and lower limit of detection [18,19]. Traditional sequencing as used throughout this article refers to a targeted NGS panel that utilizes PCR amplification to enrich for target sequences during library preparation. This assay is specifically designed for tissue and is inherently plagued with sequencing associated errors that contribute to false-positive variants at allele frequencies (AF) of 5% or lower [20]. Digital sequencing, on the other hand, utilizes hybridization capture - based approach for library preparation and incorporates unique molecular IDs to effectively obtain a lower limit of detection (LOD) of 0.1% AF. For the detection of variants in circulating cell - free DNA, digital sequencing is the superior assay due to the LOD that it achieves. However, it is more costly than traditional sequencing, and not readily available in laboratories (mostly run by the companies that designed them). Hence, our study compares traditional sequencing to digital sequencing to determine to what extent traditional sequencing can replace digital sequencing in genotyping of low frequency mutations in ccfDNA.

In this study, we subjected CRC patients' ccfDNA from plasma samples and tumor DNA from formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples, performed molecular analyses with amplicon-based NGS using Ion Torrent platform and digital NGS, and compared the variants detected from ccfDNA and tumor tissue DNA samples in order to lay the basis for its further application as a clinical diagnostic test to guide personalized therapy.

Materials and methods

Study cohort and patient selection

48 patients were selected without preference for age, gender, or ethnicity, who consented to the Assessment of Targeted Therapies Against Colorectal Carcinoma (ATTACC) umbrella protocol at MD Anderson Cancer Center [21]. All patients had histologically or cytologically confirmed metastatic CRC or unresectable locally advanced CRC documented in the ATTACC database, whose matched samples of plasma, FFPE tumor tissue, and peripheral blood mononuclear cells (PBMCs) were all available (Supplemental Figure S1). Patients were excluded if their FFPE tissue had a tumor percentage of less than 10% or if the pathology report could not be retrieved from the ATTACC database. The final cohort included 34 patients with matched tumor and plasma DNA run on the Ion Torrent platform (Supplemental Figure S2). Of these patients, 21 had data from digital sequencing platform (Supplemental Figure S3).

Tumor tissue collection and DNA extraction

The tumor tissue samples were from the same surgical cases and block numbers as those tested in our Clinical Laboratory Improvement Amendments–certified laboratory. For each patient, we obtained two slides (5-µm each) of unstained FFPE tumor tissue and one slide of hematoxylin and eosin (H&E)-stained tumor tissue. The H&E-stained slides were evaluated histologically by a dedicated pathologist to confirm the area of viable tumor tissue. Using a mapped H&E stained slides as a guide, the unstained slides of FFPE tumor tissues were microdissected, which was carried out to increase the percentage of tumor cells available for tumor DNA extraction. We extracted DNA using Arcturus PicoPure DNA Extraction Kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. The DNA slurry was further purified using Ampure beads (Beckman Coulter Life Science, Indianapolis) and eluted with low TE buffer (QIAgen, Dusseldorf, Germany) at 50 µL.

Blood sample collection and DNA extraction

For each patient, 2 aliquots of 1.4–1.8 mL of plasma and 1 aliquot of 1.4–1.8 mL of PBMCs were available for ccfDNA extraction and control DNA extraction, respectively. These samples were stored at –80 °C until they were needed for extraction. All plasma samples were handled in accordance with pre-analytical guidelines established by MD Anderson's Molecular Diagnostics Laboratory. ccfDNA isolation from plasma was performed with a QIAamp Circulating Nucleic Acid Kit (QIAgen, Dusseldorf, Germany). DNA extraction from PBMCs was performed with a DNeasy Blood & Tissue Kit (QIAgen, Dusseldorf, Germany). We followed the manufacturer's instructions for extraction and eluted the DNA to final volume of 20–50 µL.

Library preparation, sequencing, and analysis on digital sequencing platform

For digital sequencing, peripheral blood was collected in two streck tubes and shipped to Guardant Health for ccfDNA analysis using the GH360 assay. Briefly, the blood was centrifuged to separate the plasma, after which ccfDNA was extracted using the automated QIAsymphony platform. The isolated ccfDNA underwent library preparation, quantitation, and sequencing as described previously [22]. The analysis was performed on a proprietary bioinformatics pipeline at Guardant Health.

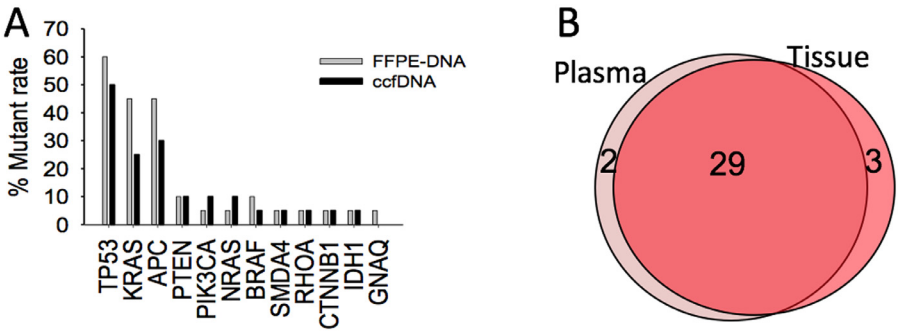

Fig. 1 (A) Distribution of variants detected in ccfDNA and FFPE tumor DNA by genes. (B) Variants detected in both ccfDNA and FFPE tumor DNA in 29 patients (25 patients had variants with an allele frequency ≥1% and four had variants with an allele frequency of <1%).

Amplicon-based NGS using ion torrent platform

Ultra-deep sequencing with amplicon-based NGS using ion torrent platform

In brief, to detect low-frequency variants, we sequenced the set of germline DNA, FFPE-DNA, and ccfDNA from two patients per run (6 samples total) for an average expected depth of 4000X. The Ion Torrent Ampliseq 2.0 kit and Oncomine Comprehensive Cancer Panel were used to generate libraries of germline DNA (PBMC), ccfDNA, and FFPE tumor DNA. Library preparation was performed using 20 ng FFPE tumor DNA, 20 ng PBMC DNA, and 6–20 ng ccfDNA. The set of germline DNA, FFPE DNA, and ccfDNA from two patients (6 samples total) were pooled together for templating on the Ion Chef per manufacturer's instructions. The prepared templates were sequenced on the Ion Proton System (Thermo Fisher Scientific) using the Ion Hi-Q PI Chip version 3 and Ion PI Hi-Q Sequencing 200 kits.

Data analysis pipeline for traditional sequencing using ion proton platform

Sequencing reads were aligned with the reference genome (human genome build 19) for base calling using Ion Torrent Suite software version 4.4.2 for the Ion Proton System (Thermo Fisher Scientific). The identification of sequence variants was facilitated by Ion Torrent Variant Caller Plugin software version 4.4-r76860, and coverage of each amplicon was determined by the Coverage Analysis Plugin software version 4.4-r77897. We used the integrative genomic viewer to visualize the read alignment, to detect the presence of variants against the reference genome, and to confirm variant calls by checking for strand biases and sequencing errors [23].

We used a custom, in-house-developed software (OncoSeek) to interface the data generated by the Ion Torrent Variant Caller with the integrative genomic viewer, to filter repeat errors due to nucleotide homopolymer regions, to compare replicate samples, and to annotate the sequencing information. OncoSeek was also used to filter out any variants in the FFPE DNA and ccfDNA that were also present in the germline PBMC-DNA. The germline PBMC-DNA was used as a control to screen out all germline variants in the FFPE and ccfDNA samples during analysis. A cutoff of 300,000 reads with a quality score of AQ20 (1 misaligned base per 100 bases) and minimum sequencing depth of 250 X were used as the measure of the successful sequencing of a sample. Variant coverage was set above 25 X for variant calling. The threshold of the frequency for variant calling was set at 5% for FFPE tumor DNA and at 1% for ccfDNA.

Statistical analysis

The Comprehensive Cancer panel used for traditional sequencing includes 143 genes, and the panel used for digital sequencing includes 68 genes. We assessed the 56 genes that both of these assays cover. Sequencing results, pathogenic variants, and their respective allele frequencies identified in ccfDNA were compared with those identified in tumor tissue and those identified in ccfDNA from digital sequencing to establish the concordance. We used Sigma plot 10.0 to prepare most of the figures and analysis. We calculated the Cohen kappa and R^2 of regression for concordance analysis of the variants detected and allele frequencies quantified. A paired *t*-test was performed for statistical analysis to compare results among data sets; it was considered as significant if $P < 0.05$ calculated by two tailed test.

Results

Variants detected in matched tissue and plasma DNA samples

We found no difference in the accuracy with which traditional sequencing detected variants in ccfDNA and FFPE tumor DNA (Fig. 1A). Traditional sequencing identified seven genes that were frequently mutated. The patient-based concordance analyses were shown in Fig. 1B by traditionally sequencing matched FFPE tumor DNA from tumor tissue and ccfDNA from plasma. Of the 29 patients for whom variants were detected in both ccfDNA and matched FFPE tumor DNA, four had variants with an allele frequency below 1% in ccfDNA (Supplemental Table S1). At an allele frequency cutoff of 1%, traditional sequencing of ccfDNA detected 92% of the variants that were detected in matched FFPE tumor DNA. We also found discordance in the numbers of variants traditional sequencing detected in each sample type; according to the assay results, three patients had detectable variants in FFPE

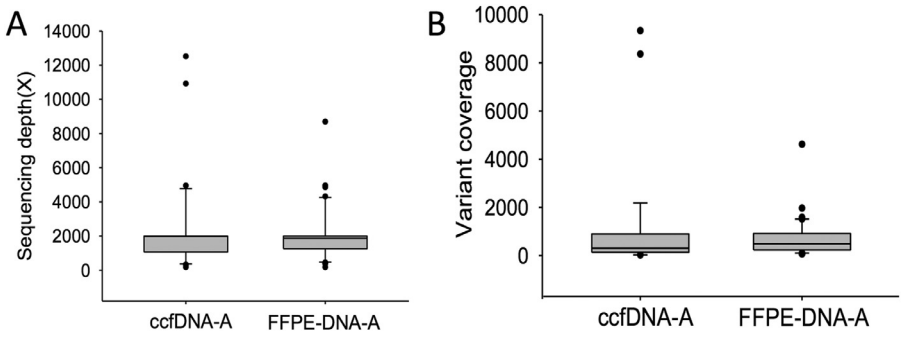

Fig. 2 Traditional sequencing depth (X) and variant coverage for ccfDNA (ccfDNA-A, refers to ccfDNA used in traditional sequencing) and FFPE tumor tissue DNA (FFPE-DNA-A). The sequencing depth (A) and variant coverage (B) of the matched FFPE tumor tissue DNA and ccfDNA did not differ significantly (*t*-test, $P > 0.05$).

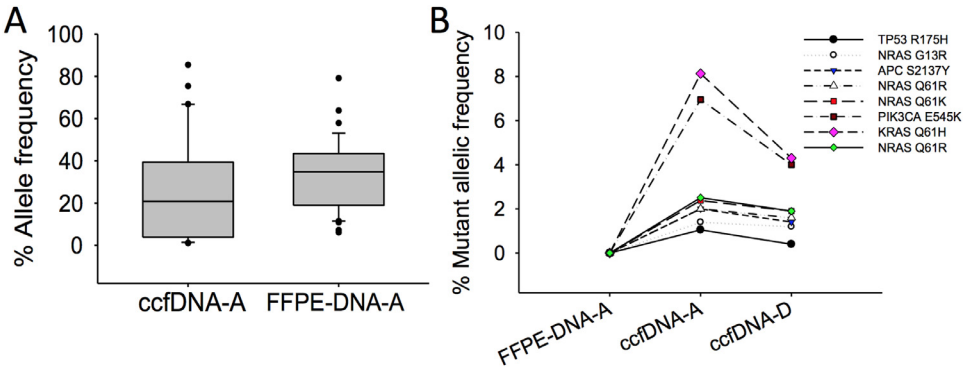

Fig. 3 ccfDNA had novel variants not seen in FFPE tumor DNA. (A) The average allele frequency of traditionally sequenced ccfDNA (ccfDNA-A) was lower than that of traditionally sequenced FFPE tumor DNA (FFPE-DNA-A), but this difference was not significant ($P = 0.12$). (B) Traditional sequencing detected variants in ccfDNA that it did not detect in FFPE tumor DNA. This was confirmed by digital sequencing of ccfDNA (ccfDNA-D, refers to ccfDNA used in digital sequencing).

tumor DNA only and two patients had detectable variants in ccfDNA only. This suggests that not all tumors shed DNA into the blood or that traditional sequencing could not detect the variants at an allele frequency cutoff of 0.1%, but did capture tumor heterogeneity better than traditional sequencing of matched FFPE tumor DNA did and identified novel variants. For example, as expected, we found no difference in the sequencing depth (Fig. 2A) and variant coverage (Fig. 2B) between FFPE tumor DNA and ccfDNA since we matched the samples together and sequenced them on one chip in the same run in order to avoid variations in sequencing coverage between the two different sample types. However, the assay detected variants at different allele frequencies in ccfDNA and FFPE tumor DNA and also detected novel clones in ccfDNA that were not seen in FFPE tumor DNA (Fig. 3).

Table 1 Concordance analysis of 21 patients for whom both traditional sequencing and digital sequencing detected variants in circulating cell-free DNA at an allele frequency cutoff of 1%.

Traditional Sequencing	Digital sequencing	
	Patients with mutation (positive)	Patients without mutation (negative)
Positive	15	0
Negative	1	5
Sensitivity	0.938	
Specificity	1	
Positive predictive value	1	
Negative predictive value	0.833	
Kappa (95% CI)	0.877 (0.6441–1)	

Abbreviation: CI, confidence interval.

Variants detected in ccfDNA on two platforms

First, we performed a concordance analysis of traditional sequencing and digital sequencing using data from 21 patients with variants detected on both platforms. The landscape of detected variants from ccfDNA focused on seven highly mutated genes as shown in Fig. 4. We performed a Cohen kappa analysis of the variants identified at an allele frequency cutoff of 1% (Table 1). The kappa index was 0.877, which indicates substantial concordance between these two platforms in detecting variants in ccfDNA.

Second, we performed a concordance analysis of the platforms using 38 variants detected in the ccfDNA of the 21 patients with variants detected on both platforms. The variant-based concordance was 91.8% at an allele frequency cutoff of 1% (Fig. 5B). Digital sequencing and traditional sequencing

Fig. 4 Landscape of variants detected from ccfDNA on two platforms**.** 7 genes were highly mutated in our patients. We evaluated the concordance of the variants detected from ccfDNA of 21 patients on these two platforms. Most of the variants can be detected on these two platforms. While, traditional sequencing with ccfDNA (ccfDNA-A) did not detect the variants with allele frequency <1% (orange). In some cases, ccfDNA could not detect the variants identified by tumor tissue DNA (red).

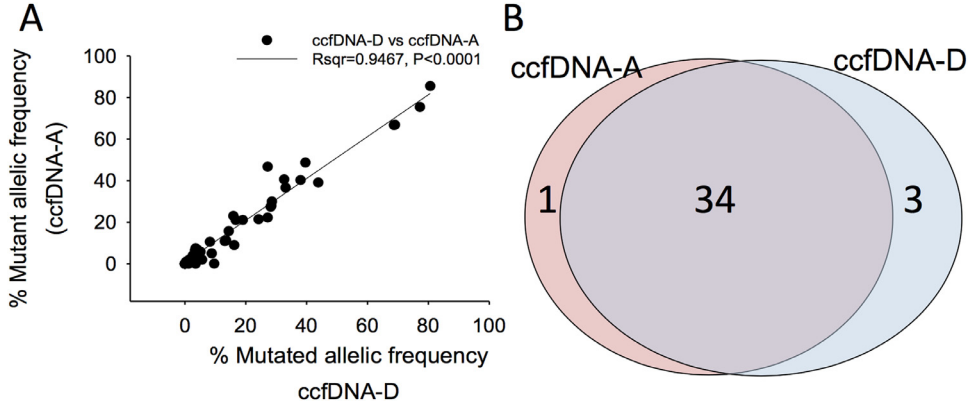

Fig. 5 (A) Regression analysis of mutant allele frequencies quantified with traditional sequencing and digital sequencing of ccfDNA. (B) Concordance of variants detected in ccfDNA between the two platforms. The assays had discordant results for 4 mutations; 1 mutation was detected in only ccfDNA with the traditional sequencing, and the other three were detected in only ccfDNA with digital sequencing. These discrepancies were confirmed by traditional NGS of FFPE tumor DNA. The mutant-based concordance was 91.8% at a cutoff of 1% AF.

using Ion Torrent plateform were discordant in their detection of four variants, all of which were detected in FFPE tumor DNA (Supplemental Table S2).

Correlation of quantified allele frequency in ccfDNA

The variant allele frequency quantified with digital sequencing, which was used as the reference, had good correlation with the variant frequency quantified with traditional sequencing ($R^2 = 0.9467$, $P < 0.001$) (Fig. 5A). The distributions of allele frequencies detected in ccfDNA by these two platforms were similar (Supplemental Figure S4), but the platforms have discordance in the variants they detected, especially in samples with low allele frequency (Fig. 6). We performed a concordance analysis for the four most frequently mutated genes in CRC (*TP53, APC, KRAS*, and *NRAS),* which revealed that these four genes' variant allele frequencies detected with traditional sequencing had good correlation with those detected with digital sequencing (Fig. 7).

Discussion

CRC is a mutation-driven disease, and a clearer understanding of how its oncogenes and oncoproteins change both over time and space is needed. Liquid biopsy–based applications enable clinical researchers to investigate tumors in ways not

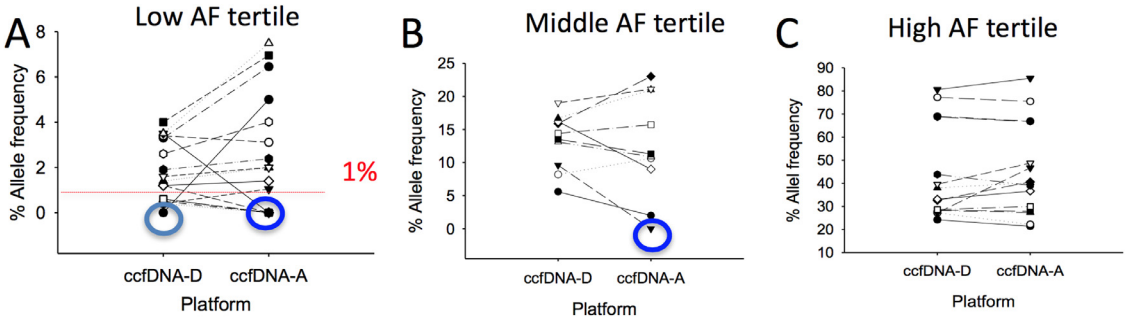

Fig. 6 Correlation of mutant allele frequency. The two platforms had similar distributions of allele frequency (C), but the mutants detected were discordant, especially in samples with low allele frequency (A, B). Note: different shaped symbol indicated the different variants with different allele frequency in the figures.

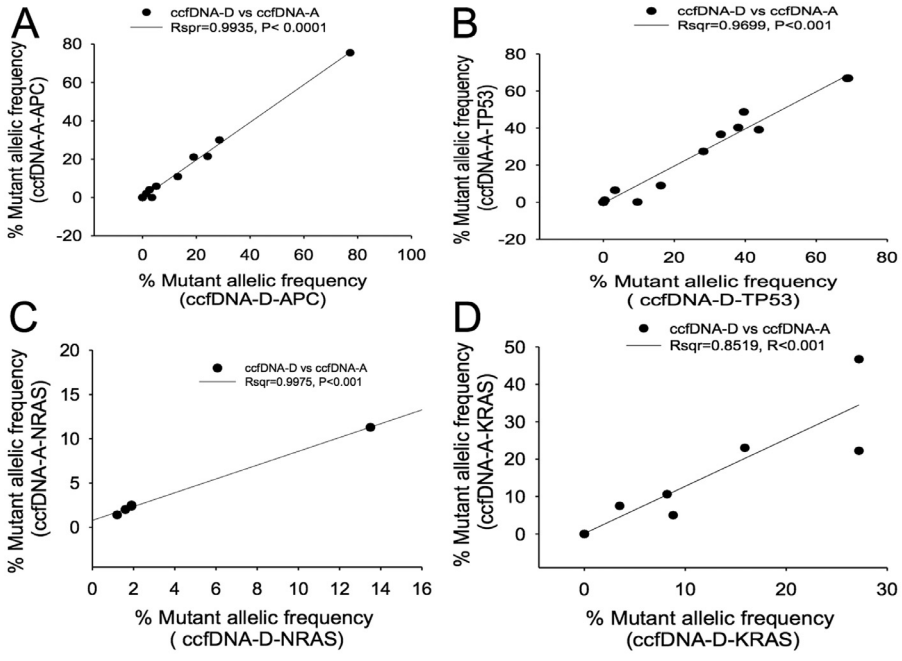

Fig. 7 Regression analysis of the allele frequencies of the four main variants detected in ccfDNA with traditional sequencing and digital sequencing. (A) *APC*; (B) *TP53*; (C) *NRAS*; (D) *KRAS*.

previously possible [10,24–28]. In the past, clinical cancer research of cfDNA relied on digital polymerase chain reaction (PCR) or droplet digital PCR applications to analyze samples with low-frequency variants [29]. However, these approaches assessed a limited number of target genes and thus limited diagnostic efficiency.

The amplicon-based NGS using Ion Torrent platform enables the detection of relevant single nucleotide variants, copy number variants, gene fusions, and indels in 143 unique genes with as little as 20ng of DNA as input. Given its merit of requiring only a small amount of DNA for the amplicon library, we compared it to the digital sequencing. Setting the digital sequencing (ccfDNA-D) as a reference [22], we found that with input of 14ng or above, the same variants were detected on both platforms. In particular, the variant allele frequencies of some of the genes most frequently mutated in CRC, such as *APC, KRAS, NRAS*, and *TP53*, had perfect correlation between the two platforms.

Traditional sequencing of FFPE tumor samples using Ion Torrent platform is well established in most clinical diagnostic laboratories, however, the allele frequency cutoff was set at 5% to avoid false positives. In the present study, traditional sequencing of ccfDNA not only detected low-allele-frequency variants present in matched FFPE tumor DNA but also detected variants that were not present in matched FFPE tumor DNA. This suggests that traditional sequencing of ctDNA would be helpful in capturing more of the heterogeneity of the tumors.

Several factors can affect the amount of tumor DNA shed into the plasma, including the tumor's volume, vascularization, and location, as well as the treatment the patient receives [22,30–33]. Sometimes, the variant allele frequency in the tumor tissue is substantially higher than that in the plasma; for example, the variant allele frequency in the tumor tissue can be more than 20%, whereas that in the plasma can be less than even 0.1%. Of 34 patients, three had variants that

were detected in FFPE tumor DNA but not ccfDNA, even with digital sequencing that had an analytic sensitivity as low as 0.1%. This indicates that the clinical utility of ctDNA for the detection of primary disease is in part limited by the biological features of certain tumors. While, ccfDNA has the potential to be used to track tumor evolution in real time, taking multiple samples throughout treatment (e.g., before, during, and after treatment) can yield insight regarding treatment efficacy and response and can help guide therapeutic strategies. Moreover, the genetic aberrations that lead to treatment resistance and/or metastasis are not likely to be represented in a primary tumor biopsy specimen obtained before the treatment starts. Thus, a strategy to detect as low of an allele frequency as possible in the ccfDNA is critically needed.

In our study, traditional sequencing detected eight variants that were discordant between ccfDNA and FFPE tumor DNA (Supplemental Table S3 and S4). Aside from the tumor itself, several conditions could have contributed to this discordance. For example, the tumor biopsy specimen could have been from a metastatic, rather than the primary tumor (#821, the tumor specimen was obtained with a liver biopsy). Or, the time between the tissue biopsy and liquid biopsy could have been substantially separated in time, allowing clonal drift (#810 and #436, the blood biopsy was performed 2 years after the tissue biopsy). Finally, the tumor may not have shed DNA into the blood (#824). Clearly, sequencing ctDNA to detect actionable mutations can benefit CRC patients with metastatic disease; however, a high level of normal ccfDNA aggravated by inflammation or injury could dilute ctDNA and interfere with ctDNA detection. So, the tumor biopsy specimen is still important because it provides known mutations for disease-specific, targeted sequencing of ctDNA.

In conclusion, amplicon-based NGS using Ion Torrent platform detected low-frequency variants in ccDNA that would not be picked up for FFPE tumor samples at the cut off of 5%. There is a high correlation between traditional sequencing on the Ion Torrent platform and digital sequencing of ccfDNA in detecting low-frequency mutations above 1%. Digital sequencing and traditional sequencing both had the substantial concordance of mutants detected and good correlation of allele frequency quantified. ccfDNA could be informative of the biological and molecular characteristics of individual tumors by identifying the low-frequency mutations. Our findings suggest that, compared with digital sequencing with ccfDNA or traditional sequencing with tumor tissue DNA only, traditional sequencing of ccfDNA can provide a faster, cheaper, broader, and less invasive assessment of cancer patients' clinical status and therapy response.

Disclosures

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2019.06.004.

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