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Validation of a Circulating Tumor DNA-Based Next-Generation Sequencing Assay in a Cohort of Patients with Solid tumors: A Proposed Solution for Decentralized Plasma Testing

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Circulating tumor DNA • Microsatellite instability status • Cancer immunotherapy

Abstract

Background. Characterization of circulating tumor DNA (ctDNA) has been integrated into clinical practice. Although labs have standardized validation procedures to develop single locus tests, the efficacy of on-site plasma-based next-generation sequencing (NGS) assays still needs to be proved.

Materials and Methods. In this retrospective study, we profiled DNA from matched tissue and plasma samples from 75 patients with cancer. We applied an NGS test that detects clinically relevant alterations in 33 genes and microsatellite instability (MSI) to analyze plasma cell-free DNA (cfDNA).

Results. The concordance between alterations detected in both tissue and plasma samples was higher in patients with metastatic disease. The NGS test detected 77% of sequence alterations, amplifications, and fusions that were found in metastatic samples compared with 45% of those alterations found in the primary tumor samples ($p = .00005$). There was 87% agreement on MSI status between the NGS test and tumor tissue results. In three patients, MSI-high ctDNA correlated with response to immunotherapy. In addition, the NGS test revealed an *FGFR2* amplification that was not detected in tumor tissue from a patient with metastatic gastric cancer, emphasizing the importance of profiling plasma samples in patients with advanced cancer.

Conclusion. Our validation experience of a plasma-based NGS assay advances current knowledge about translating cfDNA testing into clinical practice and supports the application of plasma assays in the management of oncology patients with metastatic disease. With an in-house method that minimizes the need for invasive procedures, on-site cfDNA testing supplements tissue biopsy to guide precision therapy and is entitled to become a routine practice. ***The Oncologist*** 2021;26:e1971–e1981

Implications for Practice: This study proposes a solution for decentralized liquid biopsy testing based on validation of a next-generation sequencing (NGS) test that detects four classes of genomic alterations in blood: sequence mutations (single nucleotide substitutions or insertions and deletions), fusions, amplifications, and microsatellite instability (MSI). Although there are reference labs that perform single-site comprehensive liquid biopsy testing, the targeted assay this study validated can be established locally in any lab with capacity to offer clinical molecular pathology assays. To the authors' knowledge,

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this is the first report that validates evaluating an on-site plasma-based NGS test that detects the MSI status along with common sequence alterations encountered in solid tumors.

Introduction

DNA released into the circulation by tumor cells has opened a new field of biomarker research in cancer diagnosis and treatment—in fact, it has become one of the most important areas of research in oncology [1]. Whereas normal cell turnover is the primary source of cell-free DNA (cfDNA) in healthy individuals, dying tumor cells shed a significant amount of cell-free circulating tumor DNA (ctDNA) in the blood of patients with cancer. The combination of cost-effective and high-quality deep sequencing technology to detect tumor-specific mutations, the hallmark of the ctDNA fraction, ought to advance cancer management from early detection to therapy selection and monitoring of recurrences and relapse [2, 3].

A robust cfDNA test is an important opportunity for molecular analysis in patients with cancer, yet it remains to be validated in clinical settings. In advanced disease, liquid biopsy can capture intratumoral and intermetastatic heterogeneity, thus complementing tissue biopsy to identify mutation profiles. But a range of technical, biological, and physiological factors affect cfDNA shedding, clearance, and detection, which might explain the discrepancy between ctDNA results and tumor tissue profiling [4]. Nevertheless, with the first U.S. Food and Drug Administration (FDA) approval of the cobas epidermal growth factor receptor (EGFR) Mutation Test v2 (Roche Diagnostics, Basel, Switzerland), a new era of molecular testing for cancer management started [5]. The assay tests both tissue and plasma specimens and uses real-time polymerase chain reaction (PCR) technology to identify patients with lung cancer eligible for erlotinib or osimertinib treatment. This assay and similar real-time PCR tests that detect limited genetic events are easy to validate. In contrast, next-generation sequencing (NGS) detects cancer-associated mutations in cfDNA on a wider scale and requires extensive validation [6–8]. Currently, there are two approaches for NGS-based liquid biopsy. One involves sending samples to a central laboratory [3], whereas the other one is to run an assay in-house. Recently, the FDA approved two NGS tests to analyze plasma samples of patients with advanced solid tumors: The Guardant360 CDx assay (Guardant Health Inc), and the FoundationOne Liquid CDx assay (Redwood City, California)—both tests are performed at central labs.

We retrospectively assessed the performance of a plasma-based NGS assay across paired plasma and tumor tissue samples in a cohort of patients with primary and metastatic solid tumors. The PGDx elio™ plasma resolve assay-RUO (EPR) interrogates three categories of genomic alterations in 33 genes: (a) sequence mutations, single nucleotide substitutions (SNS) and small insertions and deletions (INDELs), within coding sequences; (b) amplifications; and (c) translocations. In addition, it evaluates the microsatellite instability (MSI) status. This report describes the concordance between ctDNA and matched tumor tissue molecular profiles, discusses interpretation of observed discordant data, and illustrates applications through clinical cases. To our knowledge, this is the first report evaluating the performance of an on-site plasma-based NGS test that detects the MSI status along with common sequence alterations in the context of its clinical utility and therapeutic applications in precision oncology.

Materials and Methods

Patient Selection and Study Design

This retrospective cohort consists of 75 patients with cancer enrolled at the Englander Institute for Precision Medicine of Weill Cornell Medicine (WCM) through our institutional review board–approved protocol (WCM IRB# 1305013903). A wide range of genetic assays are performed on tissue samples to potentially guide patients' management according to current standard of care guidelines and approved clinical trials at WCM (Fig. 1A) [9–11]. Because EPR detects clinically relevant genomic alterations in 33 genes, and MSI in plasma ctDNA (Fig. 1B), we based case selection on two criteria: (a) tumor type and stage to combine primary and metastatic solid cancers and (b) gene alterations in the 33 genes covered by EPR, as well as MSI status, to allow comparison of results between tissue and plasma assays. We included tumor biopsies of primary ($n = 16$) and metastatic ($n = 59$) sites. With the exception of one patient who had four blood collections during the course of disease, all corresponding blood samples were collected within a 3-month period. This allowed us to retrospectively evaluate concordance rates of genomic profiles of primary and metastatic cancers and plasma ctDNA.

Tissue-Based Next-Generation Sequencing

We have previously reported our methods to extract and sequence DNA and RNA from tumor tissue samples [12, 13]. Briefly, all samples underwent histopathology review of either formalin-fixed paraffin-embedded tissue or fresh frozen tissue samples. DNA was extracted using the Promega Maxwell 16 MDx (Promega, Madison, WI). For whole exome sequencing (WES), a minimum of 200 ng of high-quality DNA was used and more than 21,000 genes were analyzed as previously described. Germline DNA extracted from white blood cells (buffy coat) was used as control. The WES test detects somatic sequence mutations and copy number alterations (CNAs) and evaluates MSI using MSIsensor [28]. In addition, a targeted NGS panel and whole transcriptome analysis (RNA-seq) were used to detect sequence mutations, CNAs, and translocations in a subset of cases. Total RNA was prepared in accordance with the standard Illumina mRNA sample preparation protocol (Illumina, San Diego, CA). RNA-seq and data processing was performed as previously described [29]. For fusion analysis, STAR-fusion (STAR-Fusion_v0.5.1) was used [30]. Fusions with significant support of junction reads and spanning pairs were then selected and manually reviewed. Tissue-based assays included fluorescence in situ hybridization for detecting translocations and immunohistochemistry for assessment of mismatch repair (MMR) protein expression. PCR was done to confirm the MSI status.

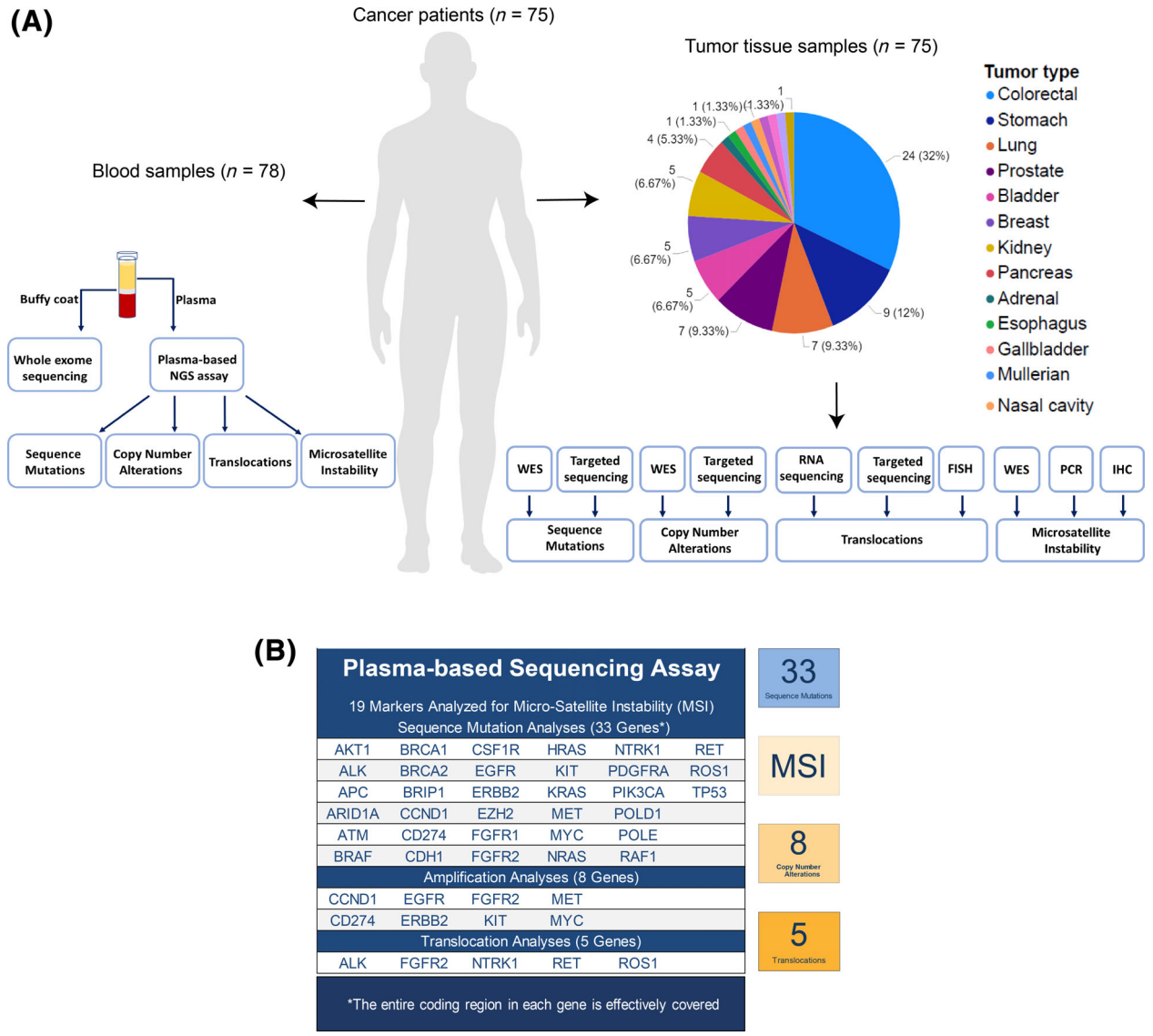

Figure 1. Molecular profiling analysis by next-generation sequencing of tissue and circulating tumor DNA (ctDNA). **(A):** Matched blood and tumor tissue samples from 75 patients with cancer were collected. Seventy-five tissue samples and 78 plasma samples from patients with 15 types of solid tumors were analyzed. Left panel: molecular profiling of tissue samples was performed using the following methods: WES to detect sequence mutations, copy number alterations (CNAs), translocations, and MSI status; RNA sequencing to detect translocations; FISH to detect translocations; PCR and IHC to detect MSI status. Right panel: cell-free DNA (cfDNA) was extracted from plasma. White blood cells fraction (buffy coat) was used as normal control for all samples tested by WES. **(B):** ctDNA genotyping was performed by PGDx elio™ plasma resolve assay-RUO (EPR) to detect the four mentioned molecular alterations: single nucleotide variants, CNAs, translocation, and MSI status.
Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; MSI, microsatellite instability; NGS, next-generation sequencing; PCR, polymerase chain reaction; WES, whole exome sequencing.

Plasma ctDNA Targeted Analysis Using PGDx EPR

cfDNA extraction from plasma samples, library preparation and libraries sequencing have been performed at the PGDx's lab. Briefly, whole blood was collected in EDTA tubes and plasma was separated from cellular components by centrifugation at 800*g* for 10 minutes at 4°C. Isolated plasma was additionally centrifuged a second time at 18,000*g* at room temperature to remove any remaining cellular debris and stored at −80°C until the time of DNA extraction. cfDNA was extracted using the Qiagen Circulating Nucleic Acids kit (Qiagen, Hilden, Germany) and quantified using the Qubit dsDNA High-Sensitivity Assay kit (Thermo Fisher Scientific Waltham, MA). After quantification, 30–40 ng of cfDNA was used to generate genomic libraries using the EPR kit. Libraries were sequenced on the Illumina NextSeq 550 (Illumina, San Diego, CA) with 150 bp paired end reads (supplemental online Fig. 1). The PGDx elio™ plasma resolve software performs an automated bioinformatic pipeline from sequence data to final variant calls, including alignment and variant calling for sequence mutations, amplifications, and translocations to identify genomic alterations in ctDNA [14, 15] and automatically filter calls to reported results [16]. The plasma assay examined one sample per patient except for one case in which four plasma specimens from different time points

were tested. Therefore, 78 plasma samples from 75 patients were analyzed.

Plasma ctDNA MSI Analysis Using PGDx EPR

Sequencing data were aligned to regions within the target panel containing mononucleotide repeats of length 14–27 base pairs in the human reference genome (hg19) using BWA-MEM [31]. A discrete peak-finding algorithm was applied to identify local maxima in the length distribution. To account for uncertainty in the repeat length determination, only peaks with at least five read pair counts, and containing ≥0.5% of the total read count at the repeat locus, were considered. If in the set of remaining peaks, the shortest identified allele was ≥3 bp shorter than the human reference repeat length, the locus was considered to be significantly shorter than the reference. In the case of the shortest allele being supported by more than 80% of the reads supporting the longest repeat allele, the locus was considered heterozygous, that is, supporting a different germline repeat length on each haplotype. Otherwise, for cases in which the shortest allele was less prevalent, the locus was classified as exhibiting instability. In the 33-gene plasma EPR panel, nine informative mononucleotide repeats were used to determine MSI status. ctDNA was classified as MSI-high (MSI-H) when two or more microsatellites were classified as MSI (supplemental online Fig. 2).

Reference Standard Sets and MSI Control Samples

Prior to retrospective assessment of the above plasma samples, we conducted a preclinical performance evaluation of the sensitivity of EPR using three different reference materials: Horizon Multiplex I cfDNA Reference Standard Set (Catalog No., Dharmacon, Inc., Lafayette, CO), a set that covers engineered single nucleotide variants (SNVs) with KRAS, NRAS, EGFR, PIK3CA, and KIT mutations at 5%, 1%, and 0.1% allelic frequencies; Seraseq ctDNA Mutation Mix v2 (Catalog No., SeraCare, Milford, MA), a liquid reference material comparable to blood; and 29 plasma samples with known MSI status (21 are MSI-H and 8 are microsatellite stable [MSS]). Of note, The Association of Molecular Pathology and College of American Pathologists provide guidelines for validation of NGS assays [17, 18].

Results

Preclinical Evaluation of the Performance of EPR

Using the Horizon Multiplex I cfDNA Reference Standard Set samples, we found 100% sensitivity detecting SNVs and INDELs in *KRAS*, *NRAS*, *EGFR*, *PIK3CA*, and *KIT* genes at variant allele frequencies (VAF) of 1%. Sequence variants in *KRAS* and *NRAS* were also found at 0.1% VAF. The assay did not detect variants in wild-type control cell line–derived DNA for any of the above genes, indicating a 100% specificity (supplemental online Fig. 3). In addition, there was 96% positive percent agreement (PPA) between EPR and droplet digital PCR (ddPCR) assays (supplemental online Fig. 3). When Seraseq ctDNA Mutation Mix v2 samples were analyzed, EPR achieved a 100% sensitivity detecting the same set of genetic variants above 0.5% VAF (supplemental online Fig. 3). We also interrogated 29 plasma specimens with known MSI status (21 MSI-H and 8 MSS). The PPA reached 80% in 15 ctDNA samples containing SNV with VAF ≥0.5%. Overall PPA for the 21 MSI-H cases was 57%, and the negative percent agreement in the 8 MSS cases was 100% (supplemental online Fig. 4). For the EPR RUO assay, the 95% limit of detection (LoD-95) for clinically relevant sequence mutations was lower than for nonclinically relevant sites as the threshold for clinically relevant sequence mutations is 0.1% VAF compared with 0.5% VAF for nonclinically relevant sites. The data indicate that the LoD-95 is between 0.5% and 0.1% VAF given the dropout of detection at the 0.1% VAF level. From a precision and reproducibility perspective, any variant reported above the LoD-95 has ≥95% confidence, whereas any variant reported between the threshold and the LoD-95 of the assay has <95% confidence. Of note, DNA input of all the three types of reference samples used was below the minimum DNA recommended for EPR (<40 ng).

Molecular Characterization of Tumor Tissue

Overall, 190 genomic alterations in tumor tissue DNA were detected in our 75-patient cohort (Fig. 2A, 2B). Each patient's tumor had at least one clinically relevant alteration that could also be detected by the EPR assay. Fifty-four cases had at least two events. The most frequent events in tissue samples were sequence mutations ($n = 157$) in the following genes: *TP53*, followed by *KRAS*, *EGFR*, *PIK3CA*, and *APC*. Thirteen patients had 15 amplifications of *MYC*, *MET*, *ERBB2*, *EGFR*, *FGFR1*, *FGFR2*, and *CCND1*. Three patients had fusions involving the *RET* or *NTRK1* genes. Tumor samples from 15 cases were classified MSI-H. Put together, these molecular characteristics of our cohort represent clinical scenarios encountered in oncology practice where molecular profiling can guide therapy.

Plasma cfDNA Yield in a Pancancer Cohort

In agreement with the literature [19, 20], cfDNA yield varied widely between patients with cancer, tumor types, and tumor burden. The range of plasma volumes was 2.4 mL to 6 mL with a median of 5 mL. Total plasma DNA yield ranged from 20 ng to 2,470 ng with a median of 60 ng. Total cfDNA yield from 16 patients with primary tumors ranged from 25 ng to 197 ng and broadly ranged from 20 ng to 960 ng in samples from 59 patients with metastases except one sample in which the yield reached 2,470 ng (Fig. 2C; supplemental online Fig. 5).

Genomic Profiling of Plasma ctDNA

The EPR assay detected 191 genomic alterations in 58 plasma samples (from 55 patients) and evaluated the MSI status for 75 patients (Fig. 2D). We found 30 events in samples from 11 patients with primary tumors and 161 events in samples from 44 patients with metastatic cancer. Number of events observed per sample ranged from 1 to 13. More than one alteration was detected in plasma of 40/44 (91%) patients with advanced disease compared with 5/11 (46%) of those without. Among the aforementioned 191 events, 158 sequence mutations were found. *TP53*, *KRAS*, *EGFR*, and *PIK3CA* were the most frequently mutated genes (Fig. 2D). EPR detected amplifications of *MET*, *FGFR2*, *CCND1*, *EGFR*, *MYC*, *ERBB2*, and *CD274* in plasma samples from 13 patients with metastatic cancers (Fig. 2D). Ten fusions involving *RET*, *ALK*, and *FGFR2* were

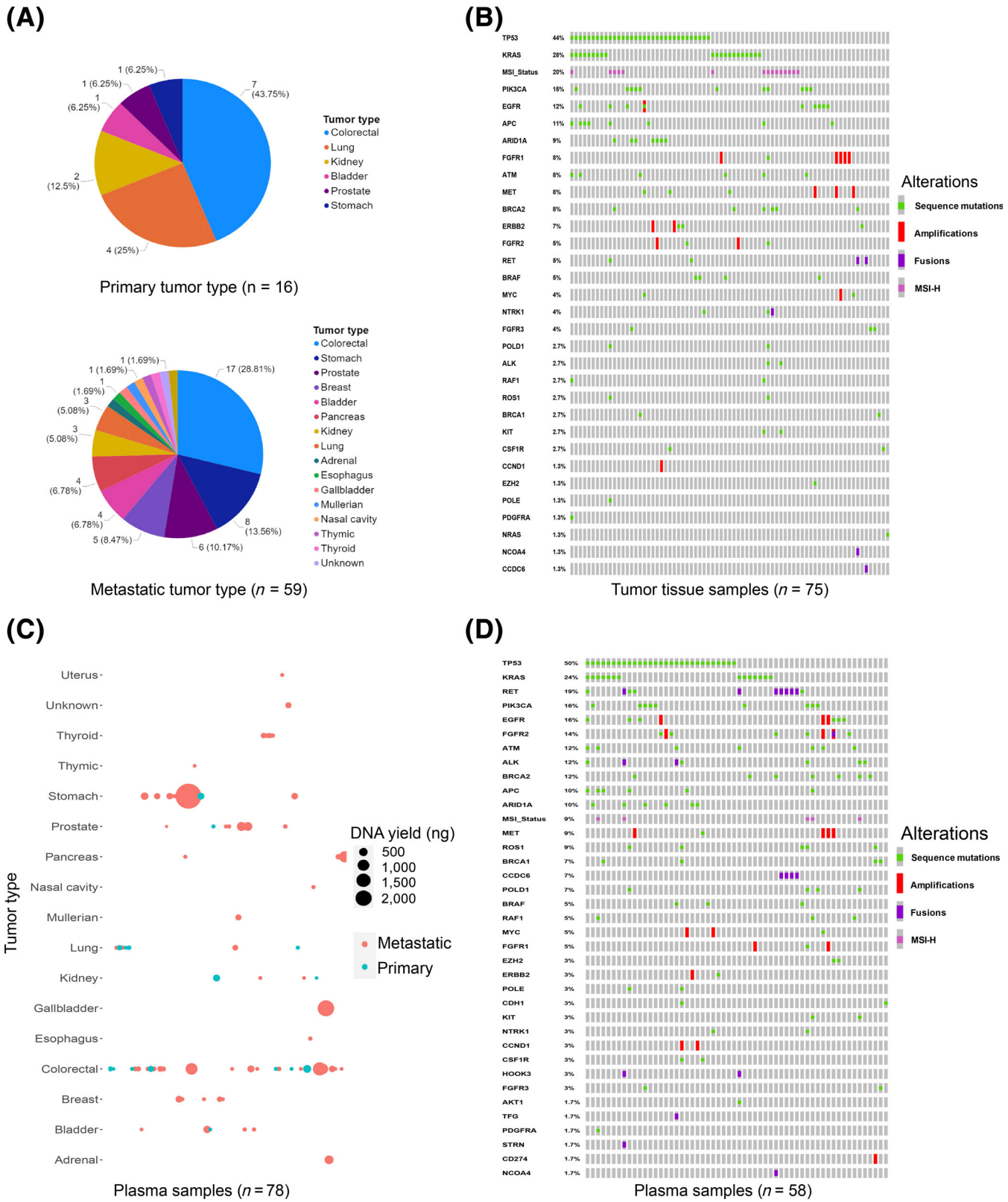

Figure 2. Mutation characteristics. **(A):** Pie charts show types of primary and metastatic solid tumors included in this study. Colorectal, stomach, and prostate cancers accounted for approximately 50% of all tumors. Matched tissue and plasma samples were obtained from 16 patients diagnosed with primary tumors and from 59 who were diagnosed with metastatic diseases. Four types of genomic alterations were identified in tumor tissue and in the cell-free DNA (cfDNA), sequence mutations, amplifications, fusions, and MSI-H. **(B):** A scatter plot shows all genetic event detected in tumor tissue. Type of alterations are represented with various colors. **(C):** Scatter plot shows cfDNA yield from plasma samples. The area of each circle corresponds to cfDNA amount. **(D):** A scatter plot shows all genomic alterations detected in plasma. Type of alterations are represented with various colors.
Abbreviation: MSI-H, microsatellite instability–high.

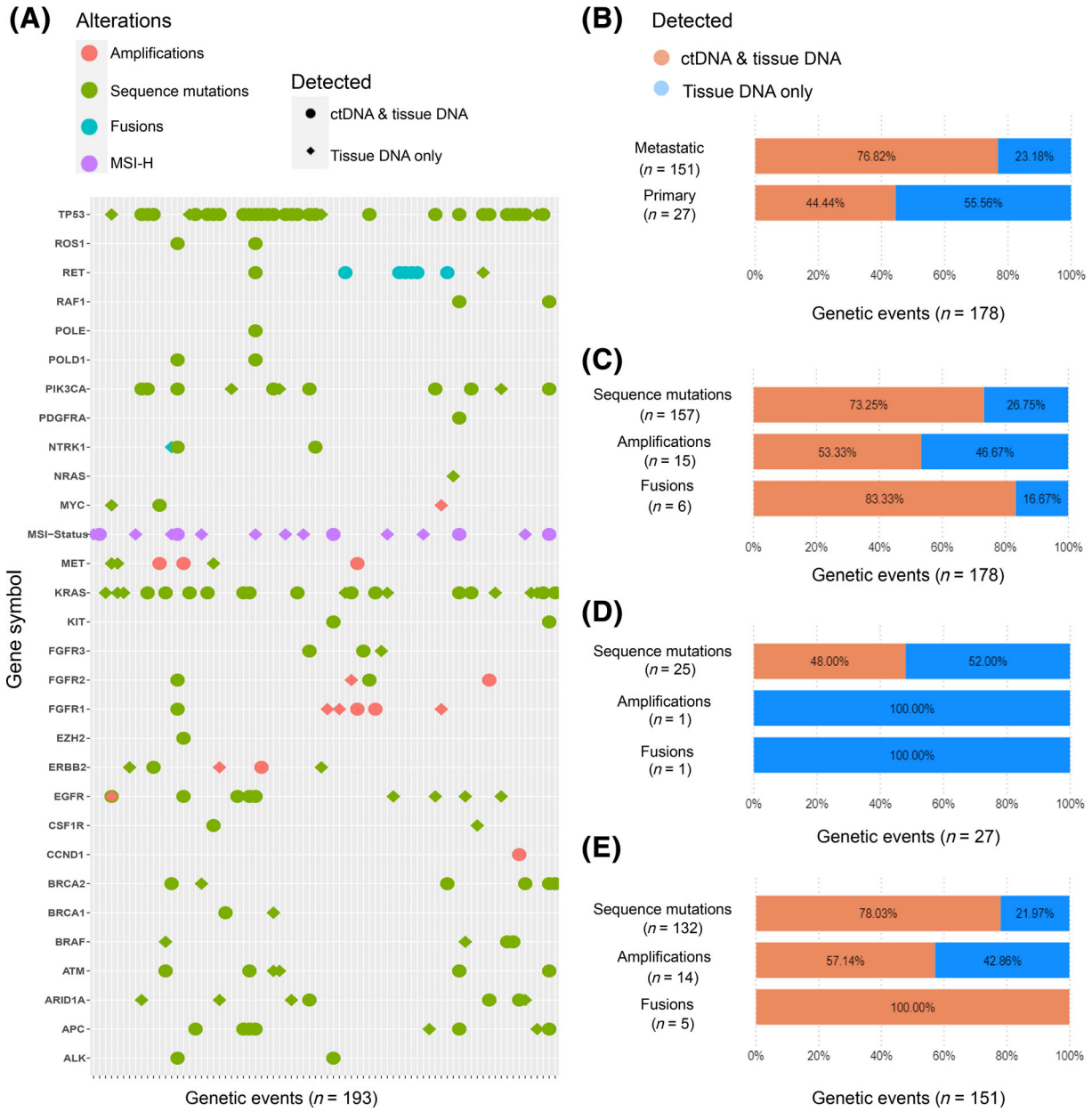

Figure 3. Concordance between tissue and plasma testing. **(A):** A scatter plot shows all events detected in both tissue and plasma samples (circle shape) or that were detected in tissue samples only (diamond shape). Type of alterations are represented with various colors. **(B):** Stacked bar charts compare percentage of concordance between tissue and ctDNA testing in primary (45%) versus metastatic diseases (77%) ($p = .00005$). **(C–E):** Stacked bar charts detail percentage of concordance regarding the three alteration types, sequence mutations, amplifications, and fusions in all samples **(C)** and in samples from primary tumors **(D)** or in samples from metastatic tumors **(E)**. Agreement between tissue and plasma testing in detecting sequence mutations was higher in the metastatic group ($p = .0002$). Abbreviations: ctDNA, circulating tumor DNA; MSI-H, microsatellite instability–high.

detected in plasma from six patients, one with primary tumor and five with metastatic disease. *RET* fusions were prevalent (7/10) and involved three fusion gene partners: *HOOK3*, *NCOA4*, and *CCDC6* (Fig. 2D).

Comparison Between Plasma DNA and Tissue DNA Analysis

Evaluation of Sequence Mutations, Amplifications, and Gene Fusions

Overall, EPR detected 128/178 expected sequence mutations, amplifications, and gene fusions found in tissue samples (Fig. 3A). The correlation between tissue and ctDNA profiling was greater in patients with advanced disease: 116/151 (77%) events reported in metastatic biopsies were detected in plasma, compared with 12/27 (45%) events present in primary tumors ($p = .00005$; Fig. 3B). Also, the range of concordant alterations per matched tissue-plasma paired samples was broader in the metastatic disease, ranging from 1 to 11, in contrast to only 1 alteration in primary cancers except for one case with 5 concordant alterations. Specifically, ERP detected 115/157 (73%) sequence mutations. The concordance of mutation

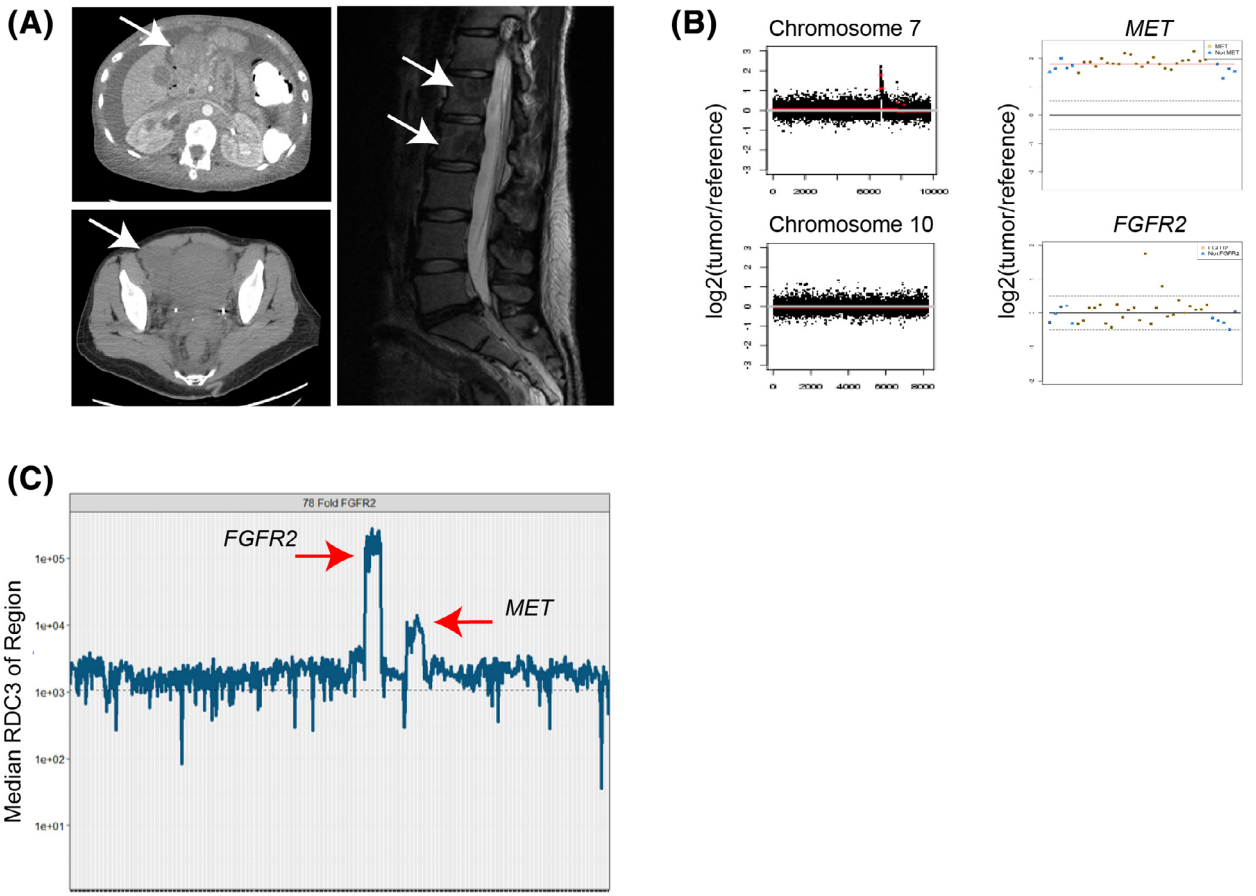

Figure 4. *FGFR2* amplification detected in plasma but not in tumor tissue. **(A):** Computed tomography (CT) imaging of metastatic gastric adenocarcinoma—with multiple metastatic sites—shows a gastric mass in the lesser curvature of the stomach with abdominal wall involvement and peritoneal carcinomatosis (upper-left image). The arrow points toward the tumor mass. The CT image in the lower-left panel reveals a pelvic mass confirmed to be a metastasis from the gastric tumor. The arrow points toward the pelvic metastasis. Sclerotic osseous metastases are seen in the lumbar spine (vertebral body of L1 and L2) (right image). Arrows point toward the bone metastases. **(B):** Molecular profiling of a tissue sample obtained from the pelvic mass was performed through two independent tissue assays, whole exome sequencing and PGDx elio™ Tissue Complete (ETC)-RUO. Both assays detected *MET* amplification, but there was no evidence for *FGFR2* amplification. The log2 score in **(B)** shows copy numbers observed at an exon-level for *MET* and *FGFR2* genes. Whereas *MET* gene is amplified, there are no significant structural copy number alterations observed for *FGFR2*. **(C):** Image of *MET* and *FGFR2* amplification coverage plot shows a high fold amplification of *FGFR2* and an amplification of *MET* in circulating tumor DNA from the patient's blood sample collected less than 15 days from tissue sample acquisition.

detection between tissue and plasma was higher in patients with metastatic disease (78%) compared with those with primary tumors (48%) across all genes ($p = .0002$; Fig. 3C–3E) and also higher in the metastatic group for the most frequent mutations (supplemental online Fig. 6).

We found an agreement in detecting 8/15 (53%) gene amplifications. Specifically, *MET*, *ERBB2*, *FGFR1*, *FGFR2*, and *CCND1* amplifications were identified in tissue and in the corresponding plasma (Fig. 3C–3E). In addition, EPR revealed an *FGFR2* amplification that was not detected in tumor tissue from a patient with metastatic gastric cancer (Fig. 4A). WES (Fig. 4B) and high-coverage targeted sequencing with PGDx elio™ Tissue Complete (ETC)-RUO (Personal Genome Diagnostics Inc., Baltimore, Maryland, United States) of the corresponding tumor were performed. Neither assay identified the *FGFR2* amplification in tissue DNA, but ddPCR on residual cfDNA from the same plasma sample confirmed the *FGFR2* amplification in blood (Fig. 4C).

The EPR assay also detected *RET* fusions in five blood samples, consistent with matching tissue results (Fig. 3C–3E). An *NTRK1* gene fusion was found in the tissue of a primary and localized colon cancer but not in corresponding plasma. An *RET-CCDC6* fusion was detected in four longitudinal plasma samples of one patient. This patient with metastatic papillary thyroid carcinoma was enrolled in our clinical research precision medicine study, which permitted us to retrospectively test serial plasma samples. Blood samples were collected over 13 months during disease course and treatment that included cabozantinib. The *RET-CCDC6* fusion was detected in the four plasma samples at an allele frequency that ranged from 0.53% to 4.55%.

Evaluation of Microsatellite Instability Status

EPR classifies tumor microsatellite status into two categories: MSI-H and MSI indeterminate, which indicates insufficient evidence of high microsatellite instability. MSI calls

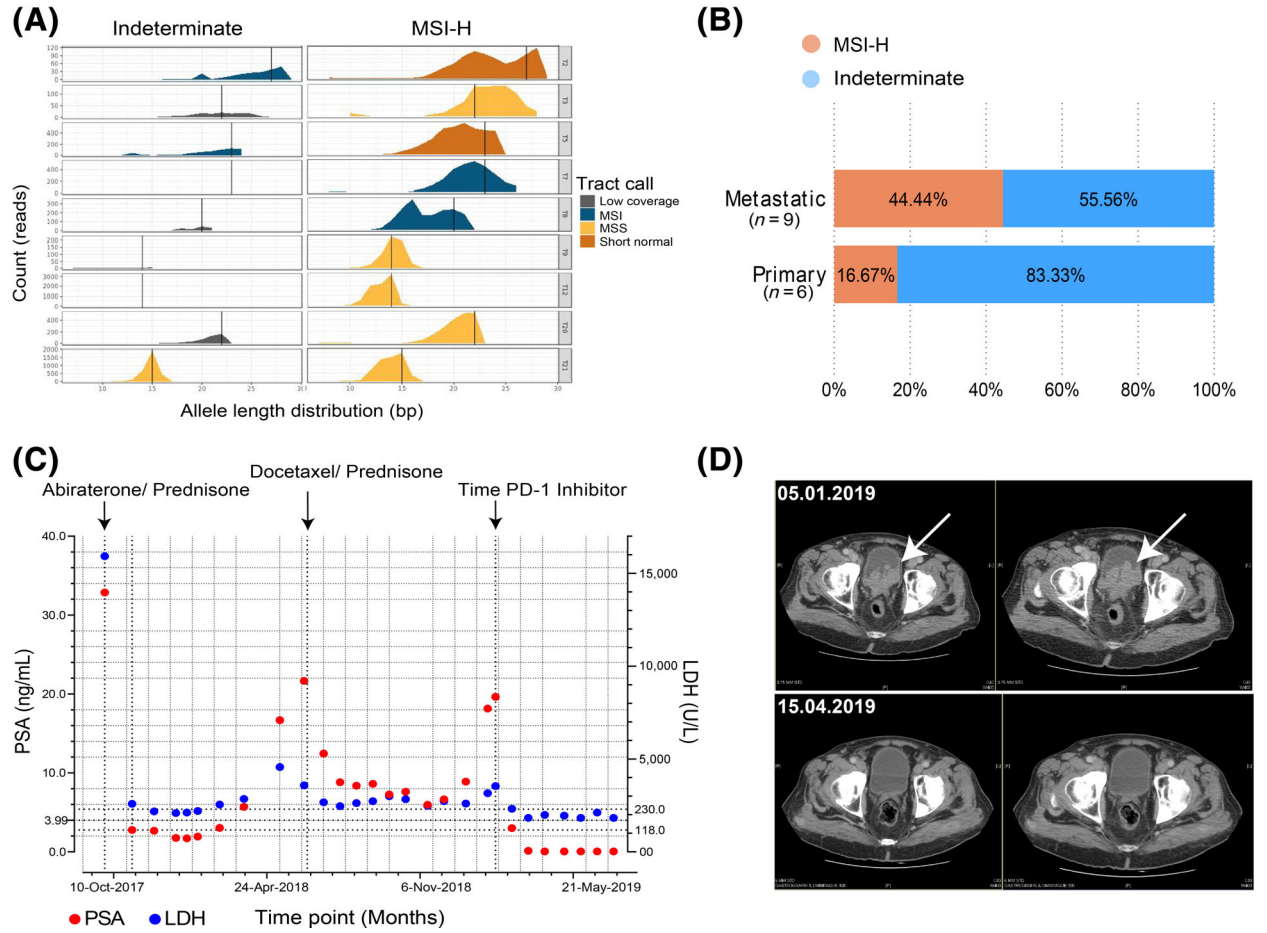

Figure 5. Plasma MSI-H status correlated with clinical and radiological response of immunotherapy. **(A):** Plots show MSI status classification based on a retrospective analysis of cell-free DNA samples. **(B):** A stacked bar chart shows the percentage of MSI-H cases detected via the plasma test compared with the results of MSI tissue testing. **(C, D):** An example of clinical and radiological response to PD-1 inhibitor in a case confirmed as MSI-H in tissue and plasma samples is presented. A patient with metastatic castrate-resistant prostatic adenocarcinoma (mCRPC) was treated with pembrolizumab (PD-1 inhibitor) for mCRPC after progression on androgen-deprivation therapy and chemotherapy. **(C):** Scatter plots show PSA and LDH levels decreased after pembrolizumab (PD-1 inhibitor) treatment and remained within the normal levels. **(D):** Computed tomography images in the lower panel reveal a prominent radiological response to pembrolizumab compared with images before starting immunotherapy (upper panel). Arrows point to tumor invasion into the bladder wall.
Abbreviations: LDH, lactate dehydrogenase; MSI-H, microsatellite instability–high; MSS, PD-1, programmed death–one; PSA, prostate-specific antigen.

from plasma and their matched tissue were concordant in 65 of 75 (87%) cases. MSI-H status was found in ctDNA of five patients, whereas the MSI status of the other 70 tumors was deemed indeterminate (Fig. 2D). All five patients who were identified to have MSI-H tumors in plasma also had MSI-H status identified in tissue. Of note, these patients had different tumor types: metastatic colon adenocarcinoma, metastatic prostate adenocarcinoma, metastatic gastric adenocarcinoma, and primary colon adenocarcinoma. EPR reported 10 cases as "Indeterminate" whereas tissue-based assays classified them as MSI-H tumors. When comparing between patients diagnosed with primary versus metastatic disease, EPR detected the MSI-H phenotype in 25% and 60%, respectively (Figs. 3A, 5A, 5B). All primary and metastatic tumors that were MSS based on tissue analysis ($n = 60$) were also classified by EPR as "Indeterminate"—that is, insufficient supporting evidence for MSI-H. Despite the moderate number of total samples studied, these results demonstrate 100% specificity, with a false positive predictive value of EPR of zero across both primary and metastatic disease.

Among the five patients with tumors classified as MSI-H by EPR, three were treated with immunotherapy based on MSI-H tissue results and had clinical response. A rather unique case of metastatic castrate-resistant prostatic adenocarcinoma (mCRPC) was found to be MSI-H based on EPR results but indeterminate based on tissue WES (MSIsensor score = 4.93). Based on immunohistochemistry (IHC) and PCR testing that identified MSI-H status, the patient received pembrolizumab after progression on androgen-deprivation therapy and chemotherapy. Clinical and radiological response was achieved after treatment with the programmed death–1 (Fig. 5C, 5D). Similarly, two patients diagnosed with MSI-H metastatic colorectal adenocarcinoma responded to cancer immunotherapy (supplemental online Fig. 7). Based on this retrospective cohort, MSI-H status detected on ctDNA

correlated with immunotherapy response in three patients with metastatic solid tumors.

Discussion

This work describes validation of an NGS plasma test that is designed to be established on-site and highlights its potential application in precision oncology practice. Until recently, most work in the field has been defined by PCR-based ctDNA analyses on specific types of cancer (e.g., lung, breast, or colon) and their associated genetic composition [21–24]. Single locus assays using quantitative PCR (qPCR) or ddPCR have been successfully applied to identify variations in the *EGFR* gene in patients with lung cancer and have received FDA approval [5]. We have expanded on that approach by assessing the EPR assay, which comprises 33 clinically relevant genes that are frequently mutated in solid malignancies. Based on various tissue testing methods, we included a list of 190 genomic alterations found in 15 different types of solid tumors in a cohort of 75 patients—each with matched tissue and plasma samples—ensuring that the established genetic makeup of this study represents those encountered in most solid tumors. It includes alterations of three mutational classes: sequence mutations (SNS or INDELs), fusions, and amplifications, as well as MSI status.

Most patients in our cohort had advanced tumors, which are characterized by increased necrosis and apoptosis and thought to release more ctDNA than patients with localized disease [20]. The cfDNA yield varied between plasma samples and was higher in the metastatic group. This increases the probability that a given sequence that harbors specific mutations is present in the tested sample and therefore is detected by a plasma NGS test. In fact, across most of our analyses, EPR had a higher performance in capturing known alterations (i.e., detected in tissue) in metastatic disease.

Compared with tissue testing, genomic profiling of ctDNA can determine intratumor genetic heterogeneity and convergent tumor evolution. Because tissue biopsies represent a sampled part of the entire tumor, possible clones residing in distant parts may be unrepresented. In contrast, plasma samples might contain ctDNA released from a greater percentage of the overall tumor mass. This makes it possible to encounter genomic alterations in cfDNA but not in tissue DNA. Those unique plasma variations were thought to be entirely because of tumor heterogeneity and clonal evolution, but recent reports have added clarification to the complex picture: such events might reflect clonal hematopoiesis [25] or simply are reported because of technical errors [26]. By analyzing replicate sets of tissue-plasma samples through four NGS-based plasma assays, Stetson et al. documented variability among results from the four plasma NGS vendors revealing technical errors or differences in sensitivities as potential causes of discrepant alterations between the plasma NGS vendors [26].

In an attempt to examine causes of disagreement between plasma and tumor tissue, we reassessed discrepant cases. For alterations detected in tissue but not in plasma DNA, tissue-specific alterations (TSAs), we reexamined the cfDNA sequencing data to verify whether those events were detected but filtered and not reported. In fact, 10 sequence mutations were detected by EPR but not reported. VAFs in (8/10) cases were detected at very low VAFs and ranged from 0.05% to 0.46%. The other two had high VAFs, 41.5% and 70.4% for *TP53* and *ARID1A*, respectively. They were considered germline variants, but this possibility was ruled out because all germline WES reads were wild type for these variants (supplemental online Fig. 8). Our data still showed disagreement, even after the reassessment. We did not detect the remaining 38 TSAs in plasma samples. Considering this broad frequency range of plasma alterations—ranging from not detected, to detected but filtered, and detected at high VAF—we infer that TSAs are encountered because not all targeted DNA sequences are released by tumor cells into blood, or they are present in plasma but at a level lower than limit of detection of the EPR assay. A common approach to test this presumption is to analyze cfDNA samples via another plasma assay, such as ddPCR. Because of the limited DNA yield and plasma availability, there was no residual cfDNA for several samples to do this analysis. Nevertheless, our data show that the overall sensitivity of EPR is comparable with other NGS-based plasma assays [9, 10, 27].

We also reviewed our WES tissue data to gain an insight on alterations that were initially reported only by EPR. A *BRCA2*_E731Gfs*19 frameshift deletion was detected in a plasma sample of a patient with metastatic carcinoma of unknown primary at a VAF of 0.72%. This 2-bp deletion was not reported by WES because only one read was detected in tumor tissue (supplemental online Fig. 9). EPR also identified a 79-fold *FGFR2* amplification, the highest fold reported in our cohort, in a patient with metastatic gastric cancer. Two separate NGS-based assays did not detect this amplification in the tissue sample. However, a ddPCR test confirmed it in plasma. This suggests that the source of the ctDNA fragment that harbors the amplification does not reside within the metastatic tissue sample analyzed and thus underlines the importance of ctDNA sequencing to complement tumor tissue testing.

We also present evidence of MSI-H detection in ctDNA and highlight its utility to inform clinical decisions in metastatic disease. It has been reported that NGS plasma assays can detect MSI-H tumors [11, 12], yet NGS-based methods to evaluate MSI status in ctDNA are underused. Whereas tissue-based testing is the standard of care approach to analyze MSI status, plasma-based methods will facilitate MSI testing across many tumor types [13]. EPR accurately captured the MSI status in 5/15 patients. In one case, the tissue MSIsensor score was below an internally established MSI-H cutoff but EPR assigned the tumor as MSI-H in agreement with two other tissue assays, PCR and IHC. More important and clinically relevant, the patient responded to immunotherapy. EPR also detected MSI-H phenotype in ctDNA of two patients who benefited from immunotherapy, supporting that MSI assessment in ctDNA has a potential application to guide treatment decisions in patients with advanced cancer. This is particularly applicable when tumor tissue is insufficient to evaluate.

Conclusion

This proof-of-concept study supports the implementation of NGS-based cfDNA assays into clinical practice as a valuable

addition to tumor tissue testing. The overall concordance between the plasma assay and tissue-based testing occurred higher in patients with metastatic disease as compared with those with localized tumors. Our effort is a step forward to a better understanding of the performance of on-site NGS plasma-based assays in clinical settings and their impact on treatment decisions. Precise identification of genomic alterations in patients with cancer requires integrating tissue and plasma assays—in our experience, a combinatorial approach proved useful.

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Disclosures

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