

Next-Generation Sequencing of Circulating Tumor DNA Reveals Frequent Alterations in Advanced Hepatocellular Carcinoma

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

Key Words. Hepatocellular carcinoma • Liquid biopsy • Circulating tumor DNA • Next-generation sequencing

ABSTRACT

Background. Because imaging has a high sensitivity to diagnose hepatocellular carcinoma (HCC) and tissue biopsies carry risks such as bleeding, the latter are often not performed in HCC. Blood-derived circulating tumor DNA (ctDNA) analysis can identify somatic alterations, but its utility has not been characterized in HCC.

Materials and Methods. We evaluated 14 patients with advanced HCC (digital ctDNA sequencing [68 genes]). Mutant relative to wild-type allele fraction was calculated.

Results. All patients (100%) had somatic alterations (median = 3 alterations/patient [range, 1–8]); median mutant allele fraction, 0.29% (range, 0.1%–37.77%). Mutations were identified in several genes: *TP53* (57% of patients), *CTNNB1* (29%), *PTEN* (7%), *CDKN2A* (7%), *ARID1A* (7%), and *MET* (7%); amplifications, in *CDK6* (14%), *EGFR* (14%), *MYC* (14%), *BRAF* (7%), *RAF1* (7%), *FGFR1* (7%), *CCNE1* (7%), *PIK3CA* (7%), and *ERBB2/HER2*

(7%). Eleven patients (79%) had ≥ 1 theoretically actionable alteration. No two patients had identical genomic portfolios, suggesting the need for customized treatment. A patient with a *CDKN2A*-inactivating and a *CTNNB1*-activating mutation received matched treatment: palbociclib (CDK4/6 inhibitor) and celecoxib (COX-2/Wnt inhibitor); des-gamma-carboxy prothrombin level decreased by 84% at 2 months (1,410 to 242 ng/mL [normal: ≤ 7.4 ng/mL]; alpha fetoprotein [AFP] low at baseline). A patient with a *PTEN*-inactivating and a *MET*-activating mutation (an effect suggested by in silico molecular dynamic simulations) received sirolimus (mechanistic target of rapamycin inhibitor) and cabozantinib (MET inhibitor); AFP declined by 63% (8,320 to 3,045 ng/mL [normal: 0–15 ng/mL]).

Conclusion. ctDNA derived from noninvasive blood tests can provide exploitable genomic profiles in patients with HCC. *The Oncologist* 2018;23:586–593

Implications for Practice: This study reports that blood-derived circulating tumor DNA can provide therapeutically exploitable genomic profiles in hepatocellular cancer, a malignancy that is known to be difficult to biopsy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide [1, 2]. Biopsy of HCC is often not performed to establish the diagnosis because the imaging is often characteristic and patients are at risk for complications from biopsy [3]. Treatment options for advanced disease are limited [4]. Sorafenib is the only systemic therapy approved by the U.S. Food and Drug Administration; it has demonstrated a median overall survival benefit of 2.3–2.8 months [5, 6]. Unfortunately, the median overall survival for advanced HCC is less than 1 year.

Molecular profiling is gaining popularity to identify genomic alterations in cancer. Traditionally, genomic sequencing is performed by extracting nucleic acids from tumor tissue. More

recently, sequencing of circulating tumor DNA (ctDNA) has become available [7, 8]. Potential advantages of ctDNA sequencing include the following: (a) ctDNA can be extracted from a vial of blood, without an invasive tissue biopsy. (b) ctDNA can be interrogated serially. (c) ctDNA can potentially harbor genomic alterations derived from DNA shed from multiple tumor sites [9]. As ctDNA sequencing is an emerging technology, the utility of clinical-grade testing in HCC has not been established. Chan and colleagues were the first to report analysis of ctDNA in HCC patients via shotgun sequencing [10]. Ono and colleagues extended these findings with polymerase chain reaction analysis of ctDNA and exosome sequencing in a

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single patient [11]. In this study, we aimed to determine if next-generation sequencing (NGS) of ctDNA from patients with HCC could identify actionable somatic molecular alterations. We include illustrative examples of treated patients and of in silico molecular dynamic simulation to propose genomic variant function.

MATERIALS AND METHODS

Patients

We prospectively evaluated 14 patients with advanced (unresectable) HCC (from January 2015 to June 2015) using a commercially available ctDNA sequencing panel (Guardant360; Guardant Health, Redwood City, CA). This study was conducted and consents obtained in accordance with University of California San Diego (UCSD) Moores Cancer Center Institutional Review Board requirements. When appropriate, patients were presented and reviewed at the UCSD Moores Cancer Center Molecular Tumor Board and UCSD Liver Cancer Group Tumor Board [12, 13]. Diagnostic radiographic findings, such as contrast-enhanced computed tomography or magnetic resonance imaging that met the liver imaging reporting and data system (LI-RADS) 5 criteria, and the presence of elevated tumor markers, such as alpha fetoprotein (AFP) or des-gamma-carboxy prothrombin (DCP), were considered in order to make a diagnosis of HCC.

Next-Generation Sequencing

Guardant360 is an NGS panel of 68 oncogenes and tumor suppressor genes utilizing Digital Sequencing of cell-free ctDNA. Complete exons are sequenced for all exons in 29 genes and critical exons (those reported as having somatic mutations in COSMIC) of 39 genes to detect and report single-nucleotide variants (SNVs), copy number amplifications in 16 genes, *ALK/RET/ROS1/NTRK1* fusions, and *EGFR* insertion/deletion mutations (supplemental online Fig. 1).

Two 10-mL vials of whole blood were collected in Streck (La Vista, NE) Cell-Free DNA Blood Collection tubes and shipped overnight at ambient temperature to the clinical laboratory improvement amendments (CLIA)-laboratory. Cell-free DNA (cfDNA) was extracted from plasma and genomic alterations were analyzed as described by Lanman et al [14].

Circulating cfDNA is mostly derived from leukocyte lysis (germline) and generally a much smaller amount of ctDNA is derived from cancer cell apoptosis/necrosis [15, 16]. All of the cfDNA fragments, both leukocyte and tumor-derived, are simultaneously sequenced. The fractional concentration, or mutant allele frequency, for a given alteration is calculated as the fraction of ctDNA harboring that mutation in a background of wild-type cfDNA. The analytic sensitivity reaches detection of 1–2 single mutant fragments from a 10-mL blood sample (0.1% limit of detection) and analytic specificity is greater than 99.9999% (15,000× average coverage depth) [14].

Definition of Actionable Alteration

A potentially actionable molecular abnormality was defined as a genomic alteration that can be impacted by an antibody with the alteration being its primary target or at low 50% inhibitory concentration by a small molecular targeted

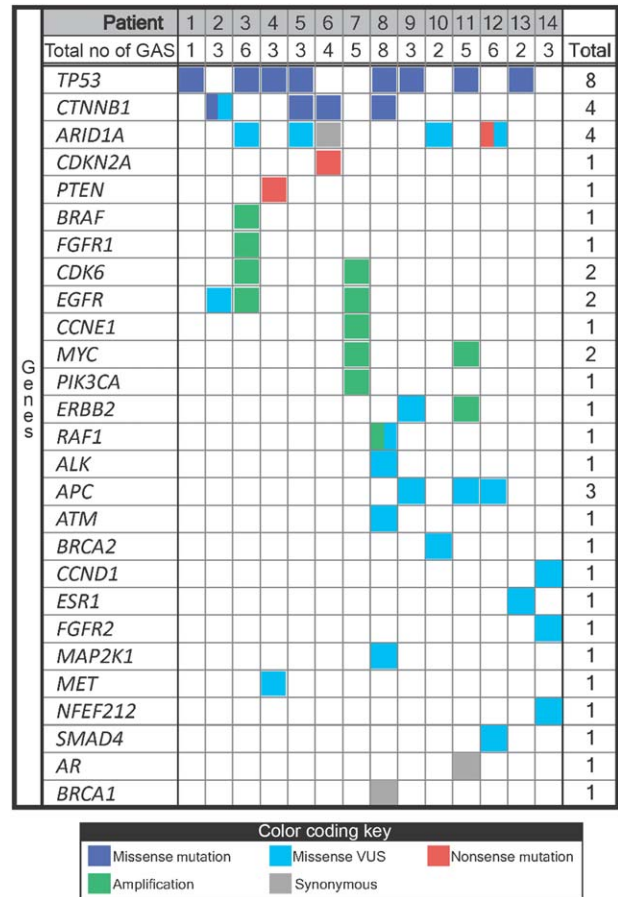


Figure 1. Results of genomic profiling from circulating tumor DNA in advanced hepatocellular carcinoma.

Abbreviations: GA, genomic alterations; VUS, variant of uncertain significance.

agent, either directly or by modulating immediate downstream effectors [17].

Molecular Dynamics Simulations

The SEMA domain of MET protein has been extracted from pdb ID 1SHY [18]. The Y501C mutant was generated by replacing Tyr501 in the wild-type (wt) structure with a cysteine. Mutated and wt protein, with disulphide bridges generated between relevant cysteine pairs, was solvated by an octahedral TIP3P water box containing ~48,500 H₂O molecules and 9 neutralizing potassium counterions. Molecular dynamics (MD) simulations were performed using the AMBER14 software package [19–21]; AMBER ff14SB force protein field and Joung/Cheatham ion parameters applied for the K⁺ counterions [22, 23]. After an initial unrestrained 10,000-step minimization, each system was subjected to several simulation steps: (a) 5 ps gradual heating from 0 to 100 K (constant volume) with restraints on SEMA domain backbone; (b) 100 ps heating to 310 K (constant pressure) with restraints on SEMA domain backbone; (c) constant pressure simulation at 310 K without restraints. Analysis of the simulation trajectories was performed using the AMBER CPPTRAJ module [19–23]. The B-factors computed from the simulations are directly proportional to the squared root mean square fluctuations of the atomic positions and therefore represent a quantification of the flexibility in the protein during the simulation.

Table 1. Demographic and baseline characteristics of 14 patients with hepatocellular carcinoma

| Variable | Patients, n = 14, n (%) |
|---------------------------|-------------------------|
| Median age, years (range) | 62 (43–73) |
| Sex | |
| Male | 12 (85.7) |
| BCLC stage | |
| B | 2 (14.2) |
| C | 8 (57.2) |
| D | 4 (28.6) |
| Child-Pugh class | |
| A | 5 (35.7) |
| B | 5 (35.7) |
| C | 4 (28.6) |
| ECOG performance status | |
| 0 | 1 (7.1) |
| 1 | 6 (42.9) |
| 2 | 6 (42.9) |
| 3 | 1 (7.1) |
| Risk factors | |
| Hepatitis B | 1 (7.1) |
| Hepatitis C | 7 (50.0) |
| Alcohol | 2 (14.3) |
| Other | 4 (28.6) |

Abbreviations: BCLC, Barcelona Clinic Liver Cancer Staging; ECOG, Eastern Cooperative Oncology Group.

RESULTS

Clinical Characteristics

With or without tissue biopsy, all 14 patients were diagnosed as having HCC (Table 1). Median age was 62 years; 85.7% of the patients were men, and 50% had hepatitis C. Child-Pugh B or C cirrhosis was noted in 64.3% of patients; 85.8% had an Eastern Cooperative Oncology Group performance status of 1 or 2 [24, 25]. The majority of patients were Barcelona Clinic Liver Cancer Staging (BCLC) C (57.2%) or D (28.6%), representing late-stage disease populations [26].

Genomic Profiling

NGS of ctDNA was successfully performed in all 14 patients (Fig. 1). All patients (100%) had somatic alterations identified (SNVs and/or amplifications; median = 3 alterations per patient [range, 1–8]) with a median mutant allele fraction of 0.29% (range, 0.10%–37.77%; analyses include variants of uncertain significance [VUS]). If VUS alterations were excluded, then 12/14 patients (85.7%) had somatic alterations identified (point mutations and/or amplifications; median = 2 alterations per patient [range, 0–5]) with a median mutant allele fraction of 1.18% (range, 0.13%–37.77%). Point mutations (excluding VUS) were identified in the following genes: *TP53* ($n = 8$ patients; 57% of all patients), *CTNNB1* ($n = 4$; 29% of all patients), and *PTEN*, *CDKN2A*, *ARID1A*, and *MET* (each $n = 1$; 7% of all patients);

Table 2. Risk factors and genomic alteration pattern in 14 patients with hepatocellular carcinoma

| Patient | Risk factors | ctDNA aberrations, ctDNA % |
|---------|--------------|---|
| 1 | HBV | <i>TP53</i> R249S 33.31% |
| 2 | HCV | <i>CTNNB1</i> S29T 0.23% <i>EGFR</i> D837Y 0.20% <i>CTNNB1</i> D32N 0.22% |
| 3 | HCV | <i>ARID1A</i> A226P 1.11% <i>TP53</i> H179P 37.77% <i>BRAF</i> amplification <i>FGFR1</i> amplification <i>CDK6</i> amplification <i>EGFR</i> amplification |
| 4 | HCV | <i>MET</i> Y501C 2.05% <i>PTEN</i> L139* 0.21% <i>TP53</i> R273C 1.18% |
| 5 | HCV | <i>TP53</i> R175H 29.51% <i>ARID1A</i> Q2207R 0.55% <i>CTNNB1</i> S33C 13.02% |
| 6 | HCV | <i>ARID1A</i> A591A 0.24% <i>ARID1A</i> T1084T 0.49% <i>CDKN2A</i> R80* 0.55% <i>CTNNB1</i> G34V 1.34% |
| 7 | Other | <i>CCNE1</i> amplification <i>CDK6</i> amplification <i>PIK3CA</i> amplification <i>EGFR</i> amplification <i>MYC</i> amplification |
| 8 | Other | <i>ALK</i> R1347Q 0.12% <i>ATM</i> R337C 0.27% <i>BRCA1</i> G511G 0.17% <i>CTNNB1</i> H36P 12.09% <i>MAP2K1</i> V60M 0.67% <i>RAF1</i> I634F 0.12% <i>TP53</i> H179R 0.13% <i>RAF1</i> amplification |
| 9 | HCV | <i>APC</i> N1919K 0.26% <i>ERBB2</i> V1128I 8.71% <i>TP53</i> H193R 0.92% |
| 10 | HCV | <i>ARID1A</i> Q171H 0.31% <i>BRCA2</i> F1219L 0.42% |
| 11 | Alcohol | <i>APC</i> T683I 2.74% <i>AR</i> A412A 0.19% <i>TP53</i> R273C 12.08% <i>ERBB2</i> amplification <i>MYC</i> amplification |
| 12 | Alcohol | <i>APC</i> S2533F 0.1% <i>ARID1A</i> Q268* 0.21% <i>ARID1A</i> E1531K 0.27% <i>FGFR2</i> G271G 0.18% <i>RAF1</i> E278K 0.23% <i>SMAD4</i> V354E 1.77% |
| 13 | Other | <i>ESR1</i> P592R 0.1% <i>TP53</i> S241F 0.13% |
| 14 | Other | <i>NFE2L2</i> F37S 0.5% <i>FGFR2</i> G570S 0.1% <i>CCDN1</i> E135K 0.1% |

Abbreviations: ctDNA, circulating tumor DNA; HBV, Hepatitis B; HCV, Hepatitis C.

amplifications were identified in *CDK6* ($n = 2$; 14% of all patients), *EGFR* ($n = 2$; 14% of all patients), *MYC* ($n = 2$; 14% of all patients), and *BRAF*, *RAF1*, *FGFR1*, *CCNE1*, *PIK3CA*, and *ERBB2/HER2* (each $n = 1$; 7% of all patients). There was no distinct pattern based on HCC risk factors (Table 2). Importantly, no two patients had the same ctDNA-derived somatic mutation pattern.

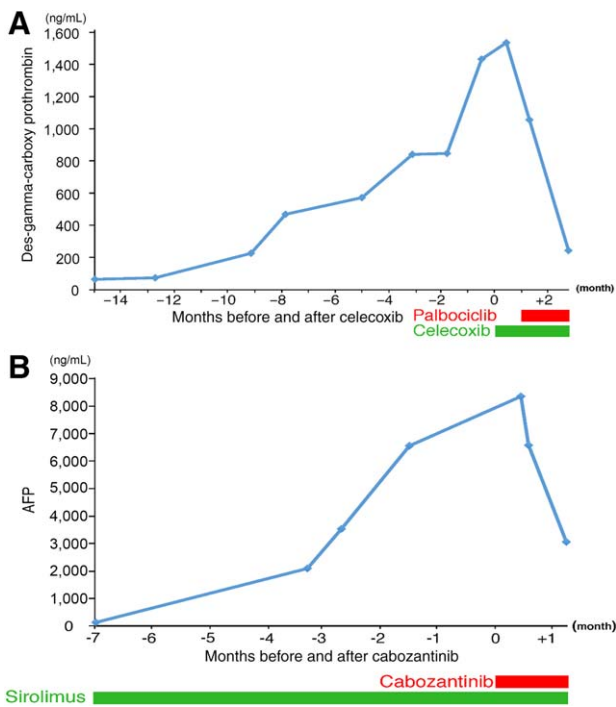


Figure 2. Clinical response to genomic alteration-matched molecular targeted therapy in two patients with advanced hepatocellular carcinoma (HCC). **(A):** See Case 1 in Results. A 62-year-old man (Patient 6; Table 2) with chronic hepatitis C-related HCC showed *CDKN2A* R80* nonsense mutation and *CTNNB1* G34V in his circulating tumor DNA (ctDNA). Patient was started on palbociclib (CDK4/6 inhibitor; CDK2A loss of function upregulates CDK4/6) and celecoxib (inhibits CTNNB1-activated pathway). Results show steep fall in levels of tumor marker des-gamma carboxy prothrombin. **(B):** See Case 2 in Results. A 64-year-old woman (Patient 4; Table 2) with chronic hepatitis C-related HCC, after liver transplant, showed *MET* Y501C missense, *TP53* R273C missense, and *PTEN* L139* nonsense mutation in ctDNA. Patient had been on the mechanistic target of rapamycin inhibitor sirolimus as immunosuppression after liver transplant. Sirolimus suppresses the pathway activated by PTEN mutations. When the MET inhibitor cabozantinib was added, AFP showed a steep decline with stable imaging. *MET* Y501C ctDNA levels also disappeared at 8 weeks (Fig. 3).

Abbreviation: AFP, alpha fetoprotein.

Therapy

Seventy-nine percent of patients (12/14) had at least one potentially actionable genomic alteration. Two of five patients (40%) treated on the basis of the ctDNA results have shown evidence of salutary effects. These two patients are described in detail. The other three patients failed to benefit.

Case 1 (Patient 6; Table 2)

A 62-year-old man with chronic hepatitis C-related cirrhosis was diagnosed with BCLC stage B hepatocellular carcinoma. He was treated with chemoembolization but developed metastatic disease approximately 2 years after the diagnosis. He was then treated with sorafenib for 2 years, after which disease progressed to BCLC stage D. Molecular profiling of ctDNA revealed the following genomic alterations: (a) *CDKN2A* R80* nonsense mutation in 0.55% of all cfDNA, and (b) *CTNNB1* G34V missense mutation in 1.35% of cfDNA. His case was discussed in the multidisciplinary Molecular Tumor Board [12, 13]. The panel concluded that the *CDKN2A* R80* mutation is likely an inactivating

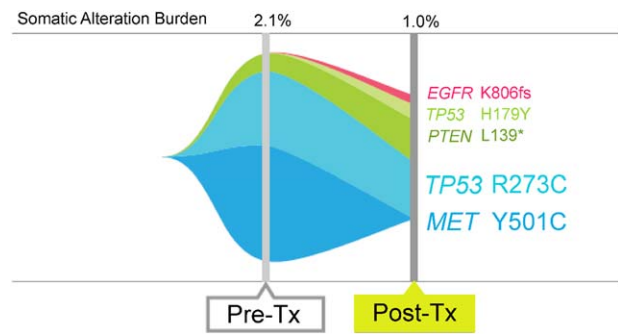


Figure 3. Genomic alteration pattern after molecular targeted therapy. The patient (see Fig. 2B) with *MET* Y501C was treated with cabozantinib (anti-MET tyrosine kinase inhibitor). Repeated sequencing demonstrated reduction in levels of the *MET* Y501C mutation.

Abbreviations: Pre-Tx, pretreatment; Post-Tx, post-treatment.

mutation because it leads to truncation of the p16INK4a protein at amino acid 80 of 156 in the p16INK4a-encoding transcript, which lacks the third and fourth ankyrin repeat that binds to CDK4, and that *CDKN2A* p16INK4a loss of function should lead to CDK4 upregulation [27]. Palbociclib is a CDK4/6 inhibitor that is available and can theoretically be used to target tumors where CDK4 or CDK6 is upregulated [27, 28]. *CTNNB1* G34V is a missense mutation, which likely activates beta-catenin and the Wnt pathway [29]. Preclinical studies have demonstrated that COX-2 inhibitors, such as celecoxib, can decrease activity of the Wnt pathway by downregulating beta-catenin expression [30]. Thus, per the Molecular Tumor Board discussion, the patient started combination treatment with palbociclib and celecoxib [12, 13].

Initially, celecoxib 200 mg by mouth twice daily was started. In 4 weeks, the DCP level declined by 31%, from 1,520 to 1,046 ng/mL (normal: 0.0–7.4 ng/mL; Fig 2A; AFP was normal at baseline). Dose-reduced palbociclib (75 mg by mouth daily, 60% of full dose) was then added to celecoxib. In the next 4 weeks, the DCP further declined to 242, an 84% decline from the baseline. Unfortunately, he developed spontaneous bacterial peritonitis, a common complication of advanced cirrhosis, and this was complicated by upper gastrointestinal bleeding and acute kidney injury [31]. Subsequently, he elected to pursue comfort care and died.

Case 2 (Patient 4; Table 2)

A 64-year-old woman with chronic hepatitis C-related cirrhosis was diagnosed with BCLC stage A HCC. Initially, she was treated with chemoembolization while waiting for liver transplant. After about 2.5 years, she received an orthotopic liver transplant. However, within 1 year, peritoneal carcinomatosis was discovered. Sorafenib was started, but she was not able to tolerate this agent due to severe facial skin rash. Her post-transplant immunosuppression was switched from tacrolimus to sirolimus because of progressive disease. Analysis of ctDNA (done at the time that the patient was classified as BCLC stage C) and liver tumor tissue were performed (the latter by Foundation Medicine, NGS of 315 genes; www.foundationmedicine.com). All the 68 genes included in the ctDNA assay are assessed in the Foundation Medicine tissue NGS panel (315 genes). Tissue biopsy NGS showed a single *TP53* R273C missense mutation. Liquid biopsy (ctDNA) revealed the following

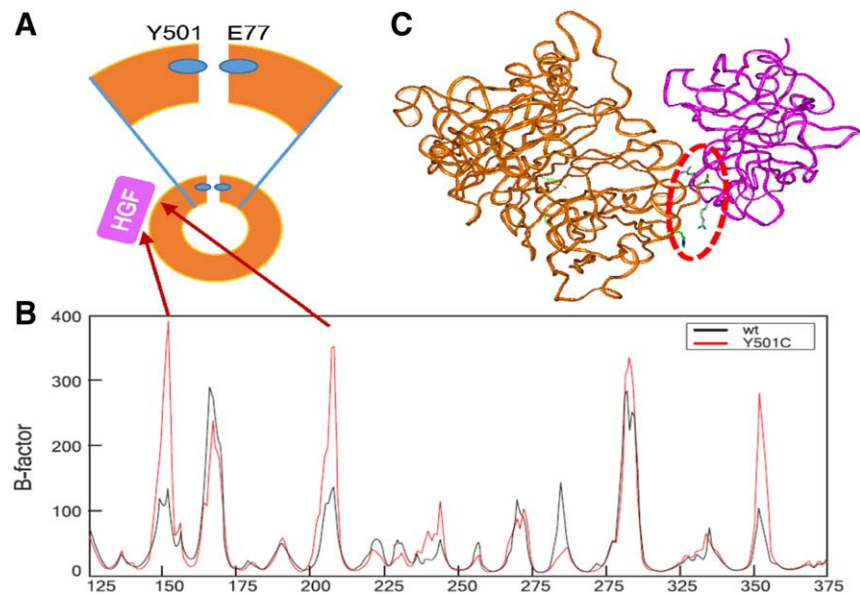


Figure 4. Flexibility analysis of Sema domain of MET. **(A):** Scheme of Sema domain. Sema domain has a ring-like shape with N and C-terminals interacting residues (lower part). The residue Y501 of the C-terminal interacts with the residue E75 of the N-terminal (blue small ovals) which probably contributes to keep this ring structure stable. Magnification of the interaction is shown on the top panel. HGF (shown as purple square) interacts with the Sema domain. The red arrows from Figure 4B to 4A indicate that the greatest increase of B-factors corresponds to residues in the HGF interacting zone of the Sema domain. **(B):** Mass-weighted B-factors for SEMA domain backbone atoms, which corresponds to the flexibility of the protein regions. Black line represents B-factor of wt MET, whereas red line represents B-factor of *MET* Y501C mutant. **(C):** Model of HGF (purple) docked to 300 ns conformer from molecular dynamics simulation of Sema domain (brown). The residues involved in the docked interface are shown in stick presentation with atomic colors (red = oxygen; blue = nitrogen; green = carbon). This region is encompassed by the red dotted oval.

Abbreviations: HGF, hepatocyte growth factor; wt, wild-type.

genomic alterations: (a) *MET* Y501C missense mutation in 2.05% of cfDNA; (b) *TP53* R273C missense mutation in 1.18% of cfDNA; and (c) *PTEN* L139* nonsense mutation in 0.21% of cfDNA. *TP53* R273C localizes within the DNA binding domain and is likely an inactivating mutation [32]. *MET* Y501C locates within the Sema domain in the extracellular region of the MET protein [18]. Polyphen score (<http://genetics.bwh.harvard.edu/pph2/>), which predicts functional importance of mutations based on three-dimensional conformation change and functional consequence, was 0.9 (range, 0.0–1.0; 1.0 indicates high likelihood of changing the function), and it is likely that the *MET* Y501C alteration is an activating mutation [33]. The fact that this *MET* gene variant is also the variant detected at the highest mutant allele fraction also supports the notion that it may be the primary driver mutation of this patient's tumor.

The patient was started on cabozantinib, a MET inhibitor, at a dose of 140 mg by mouth daily, which was later lowered to 60 mg by mouth daily due to side effects (tongue pain, fatigue, loss of appetite, and dehydration) [34]. Sirolimus (an inhibitor of mechanistic target of rapamycin, an effector downstream of PIK3CA, which is activated by *PTEN* mutations as observed in this patient) used for immunosuppression after liver transplant was continued at doses of 0.5 mg and 1.0 mg alternating every other day by mouth. Within 1 month, the AFP level declined by 63% (8,320 ng/mL to 3,045 ng/mL [normal: 0–15 ng/mL]; Fig. 2B). Imaging after 6 weeks demonstrated stable disease with some central necrosis of tumors. Repeated ctDNA analysis after 8 weeks on cabozantinib demonstrated disappearance of the *MET* Y501C mutation consistent with molecular response to therapy (Fig. 3).

Flexibility Analysis (*MET* Y501C)

TYR501 is located at the C-terminal of the SEMA domain of MET. It interacts with GLU75 of the N-terminal of this domain [35] (Fig. 4A) and most likely contributes to stabilize the domain conformation. We hypothesized that mutation of this residue to cysteine will impair the interface connection between the N- and C-terminals of the SEMA domain and would make it more flexible in some specific sites important either for dimerization or hepatocyte growth factor (HGF) binding. To explore this possibility, we conducted 300 ns MD simulations of both wt and Y501C SEMA domain of MET. We conducted analysis of flexibility of the wt and mutated proteins during MD and found that, in select sites, flexibility of the protein significantly increases due to the Y501C mutation (Fig. 4B). We found that flexibility increased in two sites at the HGF (the MET ligand) binding zone, around the residues 150 and 210. (Figure 4C, red dotted line) [35]. These regions might be involved in possible interaction of Y501C MET with HGF Figure 4C.

The importance of flexibility for protein-protein binding was demonstrated by Levy and colleagues [36], who studied complexes and showed that inhibiting protein flexibility causes slower binding and that flexibility is a necessary element in protein-protein interactions. According to the abovementioned, in our case, the increased flexibility of MET sites at the HGF binding zone might improve HGF binding. This is important because HGF stimulates the tyrosine kinase activity of c-MET [37], as demonstrated by Naldini and colleagues.

DISCUSSION

Deployment of “liquid biopsies” to detect ctDNA is an attractive concept because it circumvents the need for invasive biopsies.

This tool is especially important for diseases such as HCC, which are often not biopsied because imaging can accurately diagnose these malignancies and because biopsy carries the risk of bleeding in patients who commonly have cirrhosis, as well as the risk of seeding of the biopsy track. However, without a biopsy, assessment of the genomic profile becomes a challenge, which can be potentially addressed by a noninvasive liquid biopsy.

Our study demonstrated that all 14 patients with HCC had genomic alterations as assessed by ctDNA. The high frequency of detectable ctDNA may be related to the highly vascular nature of HCC, or possibly to attenuation in the hepatic clearance of ctDNA, because the liver provides the primary mechanism of cfDNA removal [38]. Excluding variants of uncertain significance, the median number of aberrations per patients was 2 (range, 0–5), with 86% of patients having at least one alteration. The current NGS panel completely sequenced 556 exons in a limited number of genes ($n = 68$). Conceivably, higher numbers of alterations could be found with a liquid biopsy panel targeting a higher number of genes. The median number of potentially druggable alterations was 2 (range, 0–5), with 79% of patients having at least one theoretically actionable alteration. Therefore, our study suggests that interrogation of ctDNA in this setting could have clinical utility.

There are advantages and disadvantages to use of ctDNA in blood versus tissue DNA for genomic sequencing. Advantages of ctDNA include the following: (a) serial profiling is feasible with liquid biopsies, which may therefore be deployable as a response biomarker or for monitoring of clonal evolution; (b) acquiring a blood sample is noninvasive as compared with a tissue biopsy; and (c) theoretically, ctDNA may reflect tumor DNA shed from cancer cells at multiple metastatic sites, hence illuminating intra- and inter-tumor heterogeneity [39]; this is in contrast to tissue DNA, where the sampling is limited to one part of one tumor. The latter may also explain differences between genomic profiles seen in ctDNA versus tissue (as illustrated in our patient [described in Results Case 2] who showed a *MET* Y501C in the ctDNA but not in the tissue). However, ctDNA has significant limitations as well: (a) ctDNA gene panels are currently more limited than tissue panels; (b) the amount of tumor DNA in the circulation may be quite low and therefore below the limit of detection, especially in early-stage and indolent tumors; and (c) there may be differences in the propensity of tumors to shed DNA into the bloodstream, and the factors that affect this phenomenon are still being defined. For tumor DNA to appear in the circulation, cancer cells must be undergoing significant apoptosis and/or necrosis.

In this small series of patients, ctDNA was found in all individuals, suggesting that liquid biopsy is a viable option for detecting genomic alterations in people with advanced HCC. Genetic anomalies commonly reported in HCC, including *TP53*, *CTNNB1*, and *ARID1A*, were detected in this study [40]. Furthermore, most patients (79%) had at least one alteration that was, in theory, pharmacologically tractable. Of interest in this regard, early evidence of tumor response was observed in two of five patients treated based on matching the ctDNA results to the targeted agents (Figs. (2 and 3)). This strategy merits additional investigation.

CtDNA NGS enables noninvasive interrogation of the genomic variants that drive resistance to initial lines of

treatment. Many of these variants are not identified in The Cancer Genome Atlas (TCGA) compendium because TCGA is composed of primarily stage I–II pretreated patients whose surgically excised biopsies are studied, and ctDNA is often performed on patients with advanced disease. Here we characterize a putative VUS in the *MET* gene, via molecular dynamics simulations and flexibility analysis, and suggest that *MET* Y501C has functional significance (Fig. 4). The clinical response to cabozantinib in this patient supports our modeling of *MET* Y501C as a functional driver mutation, although this tyrosine kinase inhibitor targets more pathways than just MET. Taken together, this appears to be an example of ctDNA enabling discovery of a potential new genomic target in the landscape of secondary resistance.

CONCLUSION

This study addresses the unique aspects of the management of advanced HCC. The treatment options for advanced HCC are limited, and tissue biopsy is not routinely performed. Current National Comprehensive Cancer Network guideline for Hepatobiliary Cancers (version 2.2015; <http://www.nccn.org>) states that, for patients with advanced disease, physicians may wish to consider molecular profiling to determine eligibility for clinical trials of new molecular targeted agents. However, because the liver is a highly vascular organ, and most patients with HCC are ill and have underlying cirrhosis, percutaneous needle biopsy involves a higher risk of bleeding than for most other organ biopsies and can be expensive [41, 42]. In contrast, noninvasive liquid biopsy may provide actionable genomic information without the risk of complications. An additional problem with needle biopsy-based tumor sequencing is that these small samples may have insufficient numbers of tumor cells to permit genotyping. Indeed, a recent study found that 35% of needle biopsies provided insufficient material for genomic sequencing of pancreaticobiliary cancers, whereas ctDNA was successfully detected in 100% of those subjects [9]. These findings may prove especially helpful in certain populations, such as those in Asia and Africa, with a higher prevalence of HCC (related to endemic viral hepatitis) [2].

Our study had several limitations, mostly related to the small number of patients, which precluded determining if genomic patterns differed between groups. Larger studies will be needed to address this issue. Another limitation relates to the estimation of percentage of ctDNA for mutations. Given the inability of the assay to distinguish tumor-derived wild-type alleles from normal-derived wild-type alleles, the percent given does not reflect the mutant allele fraction that would be represented in a tissue biopsy. Finally, we lacked tissue biopsies for comparison, and, furthermore, it might be of interest to determine if hepatitis C-bearing patients without HCC demonstrate ctDNA alterations; however, TCGA (<https://cancergenome.nih.gov/>) data have previously shown that some of the most common genes altered in HCC include *TP53*, *CTNNB1*, and *ARID1A*, as seen in our patients.

This report serves to also provide pilot evidence of response to biomarker-matched therapies based on ctDNA assessment in HCC. Because no two patients had the same genomic portfolio, consistent with previous reports in other cancers, individualized therapy may be necessary for optimum results [43–45]. Additional clinical utility of ctDNA may lie in its

use for serial interrogation to follow molecular response to treatment. This is illustrated in Figure 3, which shows reduction in percentage of ctDNA of a MET mutation after targeting with the MET inhibitor cabozantinib. Future studies may want to emphasize such serial blood draws in order to identify clonal evolution. ctDNA testing may therefore illuminate the atlas of genomic changes in these difficult-to-biopsy patients and provide exploitable information about therapeutic vulnerabilities. Further study of the merits of ctDNA analysis in a bigger HCC cohort is warranted.

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DISCLOSURES

Igor F. Tsigelny: Guardant Health, Inc. (C/A); **Jason K. Sicklick:** Foundation Medicine, Novartis, Blueprint Medicines (RF), Sirtex (C/A).

Kimberly C. Banks: Guardant Health, Inc. (E); **AmirAli Talasaz:** Guardant Health, Inc. (E); **Richard B. Lanman:** Guardant Health, Inc. (E). **Razelle Kurzrock:** Incyte, Genentech, Pfizer, Sequenom, Guardant, Foundation Medicine, Merck Serono (RF), LOXO Oncology, XBiotech, Actuate Therapeutics (C/A), CureMatch Inc. (OI), Roche (H). The other authors indicated no financial relationships.

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For Further Reading:

Jeffrey S. Ross, Kai Wang, Laurie Gay et al. New Routes to Targeted Therapy of Intrahepatic Cholangiocarcinomas Revealed by Next-Generation Sequencing. *The Oncologist* 2014;19:235–242.

Implications for Practice:

The recent translation of next-generation DNA sequencing technology from the research laboratory to clinical practice has enabled oncologists to personalize therapy decisions for each patient by targeting the genomic alterations driving the disease. For tumors such as primary cholangiocarcinoma of the liver, this new ability to determine all of the major genomic alterations (base substitutions, short insertions and deletions, copy number changes, homozygous deletions, and gene fusions) on very small formalin-fixed paraffin embedded clinical samples holds great promise that less toxic targeted therapies may be available for patients currently being treated with conventional “one size fits all” approaches.