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Analysis of Tissue and Circulating Tumor DNA by Next Generation Sequencing of Hepatocellular Carcinoma: Implications for Targeted Therapeutics

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Abstract

Hepatocellular carcinoma (HCC) has limited treatment options. Molecular analysis of its mutational landscape may enable the identification of novel therapies. However, biopsy is not routinely performed in HCC. The utility of analyzing cell-free circulating tumor DNA (ctDNA) by next-generation sequencing (NGS) is not established.

We performed 32 ctDNA NGS analyses on 26 patients; 10 of these patients had tissue NGS (236 to 626 genes). ctDNA was evaluated using an assay that detects single nucleotide variants, amplifications, fusions, and specific insertion/deletion alterations in 54 to 70 genes.

The ctDNA demonstrated that 23 of 26 patients (88.5%) had ≥ 1 characterized alteration, and all these individuals had ≥ 1 potentially actionable alteration. The most frequently mutated gene was *TP53* (16 of 26 patients, 61.5%). There were 47 unique characterized molecular alterations amongst 18 total gene alterations (variants of unknown significance (VUSs) excluded). ctDNA and tissue NGS frequently showed different profiles, perhaps due to length of time between tissue and blood samples (median = 370 days (range, 29 to 876 days)). Serial ctDNA evaluation in an illustrative patient treated with capecitabine demonstrated emergence of a new *TP53* alteration after progression.

In conclusion, ctDNA profiling is feasible in advanced HCC, and serial assessment using ctDNA NGS can reveal genomic changes with time. NGS of ctDNA provides a minimally invasive alternative for identifying potentially actionable gene alterations and potential molecular targeted therapies. Dynamic changes in molecular portfolio associated with therapeutic pressure in difficult-to-biopsy patients can be observed.

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Keywords

hepatocellular carcinoma; liquid biopsy; circulating tumor DNA; next-generation sequencing; molecular targeted therapy

Introduction

Hepatocellular carcinoma (HCC), the most common form of liver cancer, is caused mainly by hepatitis B or C (HVB or HVC), but may also stem from metabolic toxins such as alcohol or conditions like metabolic syndrome (1,2). Common forms of treatment for HCC include resection, transplantation, and chemotherapy (3,4). Although small, localized tumors of early stage HCC patients allow for successful treatment, individuals with advanced stage HCC are much harder to treat (3). The tumors of advanced stage HCC patients are larger and more numerous, making surgery a high-risk treatment option with increased potential for operative and post-operative complications (3). In addition, even aggressive therapies such as transplantation are associated with a high incidence of recurrence (5).

Circulating tumor DNA (ctDNA) may yield information pertinent to identifying genomic alterations in shed DNA from tumors. When necrotic or apoptotic tumor cells decompose and release DNA, ctDNA in the bloodstream can be analyzed, reflecting a so-called “liquid biopsy” (6). The ctDNA can be isolated via centrifugation, amplified and interrogated (7). In comparison to healthy individuals, patients with cancer show an increased concentration of ctDNA (8).

Liquid biopsies may be especially useful in patients with HCC because tissue biopsies may carry morbidities in these fragile patients. Herein we describe the results and clinical implications of liquid biopsies in 26 patients with advanced HCC.

Methods

Patients

ctDNA was isolated from blood samples of 26 patients with advanced HCC utilizing Guardant360, a commercially available ctDNA sequencing panel (Guardant Health in Redwood, CA (http://www.guardanthealth.com). This study was conducted and consents obtained in accordance with the University of California, San Diego (UCSD) Moores Cancer Center Internal Review Board requirements (NCT02478931). The UCSD Moores Cancer Center Molecular Tumor Board and UCSD Liver Cancer Group Tumor Board were used as resources for sharing and reviewing patient cases when deemed necessary.

Next-Generation Sequencing

Liquid biopsies—The digital sequencing required for this study was conducted in a College of American Pathologists (CAP)-accredited and Clinical Laboratory Improvement Amendment (CLIA)-certified clinical laboratory. Liquid biopsies require two 10mL samples of blood, which collectively provide 5ng of DNA for ctDNA analysis. Liquid biopsy panel had each base sequenced at average raw coverage depth of 8,000× with a minimum average

base coverage of 3,000× and a minimum Qscore of 20; Tissue biopsy coverage depth was 500×. The lowest detection mutation fraction is 0.1% for ctDNA and 5% for tissue biopsy. Detailed methods for both liquid biopsy ctDNA and tissue NGS have been previously published (9,10). There were 32 blood specimens in the study (N = 26 patients). A 54-gene panel was used for one sample; a 68-gene panel for 28 samples; and a 70-gene panel for 3 samples (Supplemental Tables 1, 2, and 3). One patient had 3 blood samples collected, four patients had 2 blood samples collected, and the remaining twenty-one patients had a single blood sample.

Tissue molecular profiling—In this study, tissue NGS was performed in 10 patients. In nine patients, a 236-gene panel was utilized (FoundationOne™, Cambridge, Massachusetts, http://www.foundationone.com); one patient, a 626-gene panel (Molecular Health, http://www.molecularhealth.com).

Definition of Potentially Actionable Alterations

A potentially actionable alteration is defined as a genomic alteration which produces a protein product that may directly serve as the primary target of an antibody, or the target, at low 50% inhibitory concentration (IC50), of a small molecule inhibitor. If an immediate downstream effector of a gene product alteration can be modulated, that gene was also considered potentially actionable. Finally, gene products that can be targeted because of their differential expression in tumor versus normal cells were considered actionable, regardless of impact on function.

Results

Patient Demographics

Twenty-six patients with HCC were analyzed (20 (76.9%) men) (Table 1). Their median age was 65 years (range, 44 to 74 years). Risk factors for HCC included HBV, HCV, alcohol, and metabolic syndrome, with HCV being the most common (17 patients (65.4%)). Regarding Barcelona clinic liver cancer (BCLC) staging (11), stage C was the most frequent (46.2%). Child-Pugh classification (12) was most often Stage B (50.0%), and patients most commonly had an ECOG (13) performance status of 2 (42.3%).

Genomic data was collected on the 26 patients via liquid biopsy for every patient and tissue biopsy for 10 (38.5%) of the patients (Figure 1A and B, Table 2). In 9 of the 10 patients with both tissue and liquid biopsy, the tissue predated the liquid biopsy (Figure 2).

Genomic testing and alterations found in ctDNA

In total, alterations in 37 different genes were observed, including characterized alterations and VUSs. Only 18 different genes were involved at least once with a characterized alteration. The median number of genes with characterized alterations per patient was 1.5 (range, 0 to 5). Twenty-three of 26 patients (88.5%) had at least one characterized altered gene; the three patients without characterized alterations had VUSs. In total, there were 47 distinct characterized molecular alterations amongst the 23 patients with characterized anomalies.

The most common genetic alterations (including variants of unknown significance (VUSs)) occurred in the *TP53* gene, a master regulator of apoptosis and the cell cycle, affecting 16 (61.5%) of the patients. The second most common alteration affected the *CTNNB1* gene (8 (30.8%) of the patients), a key regulator of the Wnt pathway (Figure 1A). *ARID1A* is a subunit of the SWN/SNF complex, an epigenetic regulator, and was altered in 23.1% of patients (N = 6). The rest of the genomic alterations occurred at low frequency and affected oncogenes and tumor suppressor genes such as *EGFR, MYC, APC, ATM, CDK6, ERBB2, RAF1, BRCA1, FGFR1, KRAS, PIK3CA, ALK,* and *BRAF*. A visual breakdown of all observed genetic alterations and the patients with whom they corresponded has been provided (Supplemental Figure 1).

Thirteen patients (50.0%) had a genomic portfolio that was identical to at least one other patient: 6 patients with only *TP53* gene alterations; 2 patients with only *CTNNB1* gene alterations; 2 patients with only *TP53* and *CTNNB1* gene alterations; and 3 patients that had no characterized genomic alterations. However, no patients were identical at the molecular level, since patients with, for instance, *TP53* anomalies, had distinct loci mutated.

Percent ctDNA found in liquid biopsies and correlations with AFP and Child Pugh class

The highest mean mutant allele frequency of ctDNA was seen in *TP53* mutation (N = 16 patients; mean ± standard error (SE) = 12.0 ± 4.0%) (Figure 1B). This was followed by *CTNNB1* with 11.1± 4.9% (N = 7 patients). Other genes that had mutant allele frequency of greater than 1.0% were *MET* and *NFE2L2*; all other characterized genomic alterations had less than 1.0% mutant allele frequency of ctDNA.

Percent ctDNA correlated well with AFP ($P < 0.001$). We did not find a significant correlation between Child Pugh class and %ctDNA, perhaps because of the small number of patients in each subgroup (Table 2).

Potential actionability: Examples of possible targeted therapies

Among altered genes that included characterized alterations (N = 18 genes), 16 (88.9%) were potentially actionable (Table 3). All 23 patients (88.5% of 26 patients studied) had at least one potentially actionable alteration in their ctDNA. The median (range) number of potentially actionable ctDNA alterations was 1.5 (0 to 5). For example, CTNNB1 encodes beta catenin, a key regulator of the Wnt pathway. Sulindac and celecoxib demonstrate inhibitory activity against this pathway; furthermore, experimental therapies are in development (NCT02675946; CGX1321). Multiple agents, such as erlotinib and cetuximab, are currently approved by the Food and Drug Administration (FDA) for targeting the EGFR receptor tyrosine kinase. CDK6 is a regulator of cell cycle that may be impacted by palbociclib, a CDK 4/6 inhibitor.

Comparison of ctDNA and Tissue NGS Results

Ten patients had both tissue and ctDNA NGS testing and are shown on Figure 2. Nine patients (Table 2, **cases #3, 4, 7, 10, 14, 17, 19, 21, 23**) had characterized alterations in either their tissue NGS, ctDNA NGS, or both. One patient had no characterized alterations in either tissue or ctDNA NGS (Table 2, **case #16**). Examining alterations that were assayed in

both the tissue and ctDNA NGS, six genomic alterations were found in both tissue and ctDNA; 18 alterations were found only in ctDNA; and 14 alterations were found only in tissue. The median time interval between tissue and liquid biopsy for ctDNA for the ten patients was 370 days (range, 29 to 876 days). The concordance for the three most commonly altered genes was as follows: *TP53*, 50.0%; *CTNNNB1*, 100.0%; and *ARID1A*, 90.0%.

Serial analysis of ctDNA and monitoring of treatment effect

A total of five patients (19.2%) had multiple liquid biopsies for ctDNA performed, with a median of 133 days between tests. The percent ctDNA for alterations varied between test dates. Further, only one of the five patients with multiple tests had an identical portfolio of genomic ctDNA alterations between tests. These patients were on therapy between tests.

ctDNA can be measured at multiple time points during the course of treatment. An example in Figure 3 demonstrated that the alteration profile changes over time. This patient was diagnosed with BCLC Stage B HCC and received multiple locoregional therapies. The patient also started sorafenib; however, his disease metastasized to regional lymph nodes. Initial analysis of ctDNA revealed two genomic alterations: *BRCA2* F1219L and *ARID1A* Q171H. The patient started metronomic dose capecitabine (14). The following ctDNA analysis, which occurred after capecitabine treatment was stopped due to toxicity and progression, demonstrated reduction of mutant allele fraction in two VUSs: *BRCA2* F1219L (0.42% to 0.12%) and disappearance of *ARID1A*Q171H alteration (0.31% to 0%). However, there were two new aberrations emerged: *BRCA1* R866C (VUS) and *TP53* A138V (characterized alteration). Because of the patient's deteriorating general condition and increased bilirubin, the patient chose hospice care.

Discussion

This study investigated ctDNA analysis in 26 patients with advanced HCC. Twenty-three patients (88.5%) had at least one characterized alteration, and, in each of these individuals, there was at least one potentially druggable alteration. This observation suggests the potential clinical utility for ctDNA assessment. This utility may be especially relevant in HCC because biopsies are often not performed for diagnosis, at least in part because of their potential for complications.

In total, there were 47 distinct characterized molecular alterations involving 18 genes amongst the 23 patients with characterized anomalies. ctDNA was, however, detected in all 26 patients, albeit only VUSs were seen in three of the patients. This rate of detection is comparable to that in a study of patients with lung cancer that showed that ctDNA alterations were discernible in 83% of participants (15).

The most common alterations in our study were in *TP53, CTNNB1* and *ARID1A*. The literature suggests that these genes are also commonly found to be abnormal by tissue NGS of HCC (16). Four of five patients with metabolic syndrome, 10 of 17 patients with HCV, and the one patient with HBV had a *TP53* mutation (Table 2). Previously, HBV-related HCC has been associated with *TP53* mutations (17). Other genes that were occasionally aberrant

(characterized alterations) and of possible interest from a treatment point of view include *EGFR, ERBB2, PIK3CA/PTEN, CDK4/CDKN2A*, and *BRAF* as well as *KRAS* (Figure 1A, Table 3)(16,18). Indeed, 16 of the 18 genes with characterized alterations were potentially pharmacologically tractable. Clinical utility of these treatment options in genomically-matched patient populations is currently being investigated in basket trials (e.g., NCI-MATCH (NCT02465060), ASCO TAPUR (NCT02693535)). Of interest, however, no two patients were identical at the molecular level. This observation may complicate the use of genomics for choosing therapy, at least in traditionally designed trials. Ultimately, treatment choices may need to be individualized.

The highest percent ctDNA was seen with *TP53* mutations (mean ± standard error (SE) = 12.0 ± 4.0%) (Figure 1B). This observation may be significant because *TP53* overexpression is an strong indicator of poor prognosis for HCC patients (19). The second highest percent ctDNA was seen with *CTNNB1* at 11.1± 4.9%. All other genes had a percent ctDNA of less than 2.0%. Previous studies have suggested that higher percent ctDNA correlated with a worse prognosis (20).

We attempted to determine if higher AFP levels correlated with higher percentage ctDNA. Percent ctDNA correlated well with AFP ($P < 0.001$). This may not be surprising since both percent ctDNA and AFP correlate inversely with prognosis (21). We did not find a significant correlation between Child Pugh class and %ctDNA, perhaps because of the small number of patients in each subgroup.

A comparison of liquid and tissue NGS was also performed in this study. Data from ctDNA NGS was collected in all 26 patients, while data from tissue NGS was collected in only 10 of the patients (38.5%). The concordance levels for the three most commonly altered genes, *TP53, CTNNB1,* and *ARID1A,* are 50.0%, 100.0%, and 90.0%, respectively. These concordance rates are similar to those in previous studies that compare tissue and ctDNA (22). On the other hand, of alterations evaluated in both tissue and ctDNA assays, 18 alterations were found only in ctDNA NGS and 14 alterations were found only in tissue NGS. The median time between tissue and ctDNA sequencing was 370 days (range, 29 to 876 days), during which many new genomic alterations could have occurred. Previous reports indicate that the concordance of tissue and ctDNA sequencing depends on the time interval between two tests (the longer the interval, the less the concordance) consistent with the notion of genomic evolution (20). Other reasons for discrepancies between ctDNA and tissue genomics include technical factors, suppression of ctDNA clones as a result of therapy, or due to the fact that ctDNA includes shed tumor DNA from multiple sites, while tissue includes genomic alterations in only the site biopsied.

ctDNA may also change with time under therapeutic pressure. For instance, prior results have shown that either urine or blood ctDNA may be suppressed by therapy (18). In our patient, dynamic change of ctDNA mutant allele fraction can be seen as demonstrated in Figure 3. Serial ctDNA analysis captured dynamic molecular events wherein *ARID1A* and *BRCA2* VUS mutant allele fraction decreased on capecitabine while *BRCA1* VUS and *TP53* characterized alterations emerged (with the follow up ctDNA assessed after patient

was taken off drug due to progressive disease), perhaps reflecting the effects of the chemotherapy on various cancer clones.

There are some limitations to this study. First the ctDNA panel grew bigger with time (Supplemental Tables 1, 2, and 3). However, the most commonly altered genes were seen in the original 54 gene panel (*ARID1A b*eing an exception). On the other hand, of the 32 ctDNA tests performed, only one sample was tested with the initial 54 gene panel; 28 samples were tested with the 68 gene panel; and three samples, 70 genes. Second, the number of patients in our study is relatively small. Future studies of larger numbers of patients are warranted. Finally, serial ctDNA sampling is required in more patients. Although one of our patients showed emergence of new ctDNA alterations under therapeutic pressure (Figure 3), in other patients, treatment was performed at outside institutions and correlations with serial sampling were not possible. Additional studies should focus on large serial sampling studies are of interest.

In summary, our observations suggest that ctDNA analysis via liquid biopsy is feasible in HCC and frequently reveals diverse, potentially actionable genomic alterations. Dynamic changes in ctDNA after treatment can also be observed with serial assessment. Further investigation of ctDNA clinical utility in larger cohorts of patients and correlation with therapeutic outcome is warranted in this difficult-to-biopsy cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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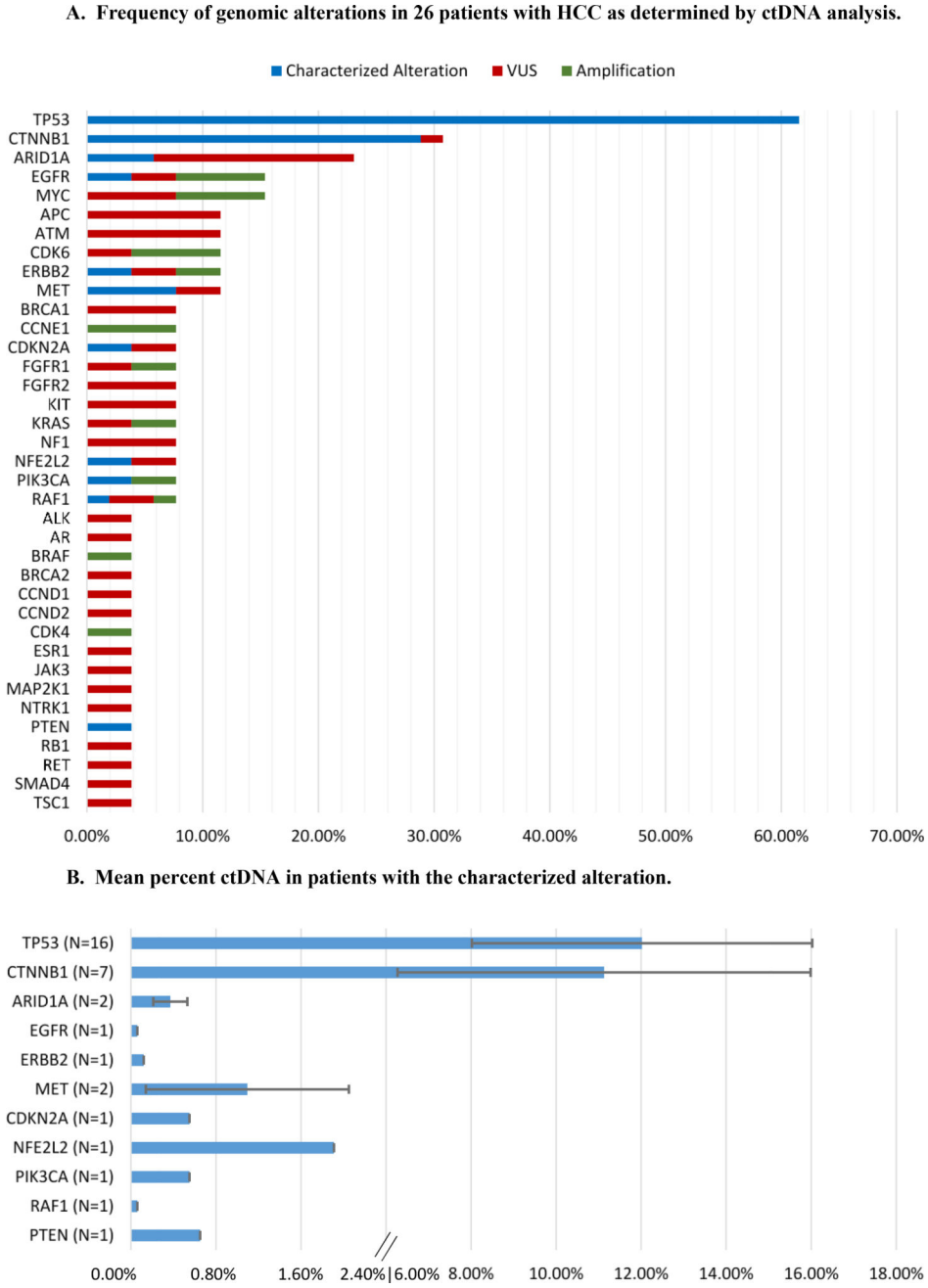

Figure 1.

A: Frequency of genomic alterations in 26 patients with HCC as determined by ctDNA analysis. Chart shows the percent of patients with the alteration.

Abbreviations: ctDNA = circulating tumor DNA; HCC = hepatocellular carcinoma; VUS = variant of unknown significance

B: Mean percent ctDNA in patients with the characterized alteration. N represents the number of patients with the alteration. The mean ± SE of %ctDNA is depicted. Only patients with non-zero results for each particular characterized alteration were included in the

calculations for that given alteration. Percent ctDNA is calculated as a fraction of cell free DNA.

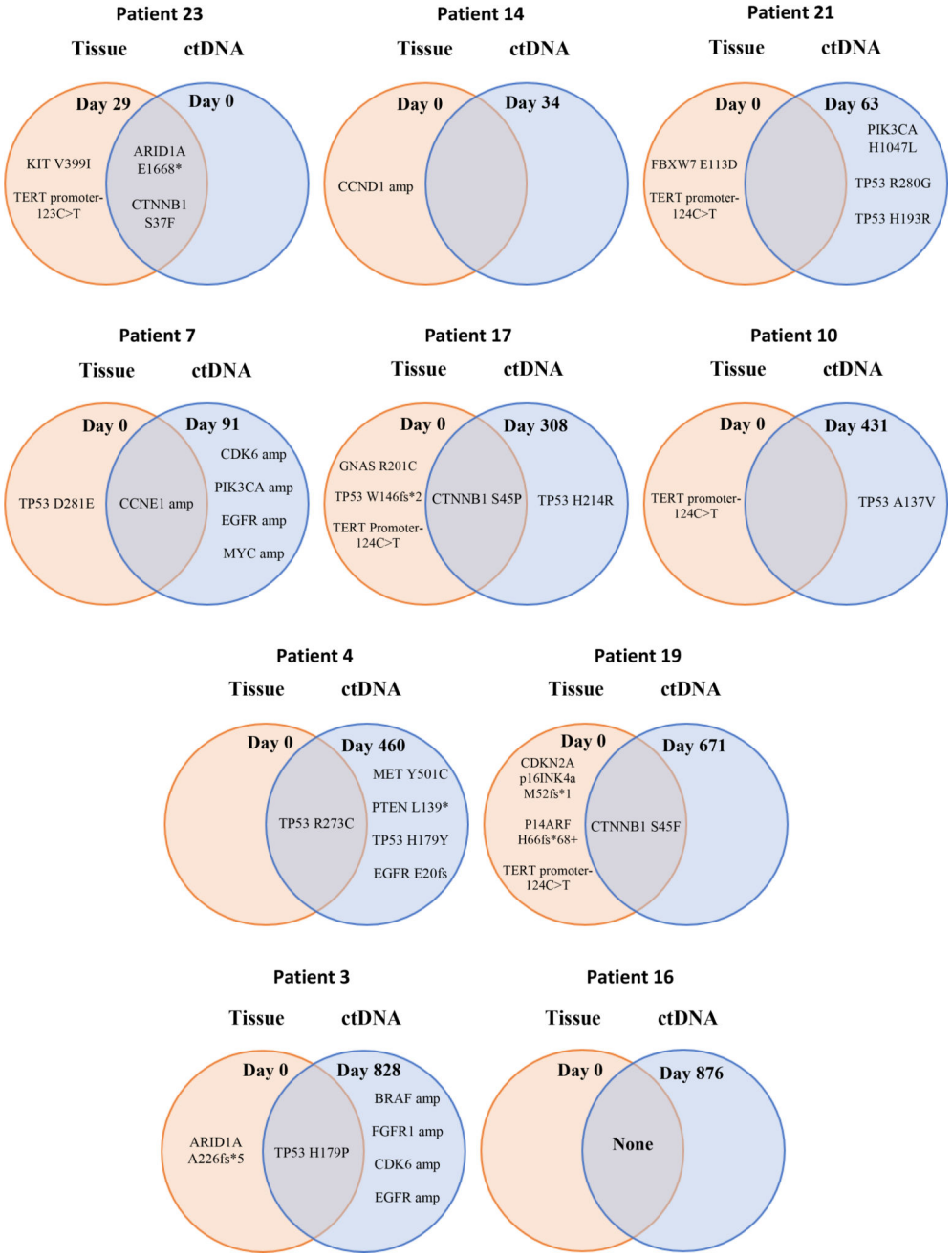

Figure 2. Venn diagrams showing the overlap between tissue NGS and ctDNA NGS for characterized genomic alterations

The relative dates between the collection of the tissue NGS sample and ctDNA sample are also presented with Day 0 representing the date of the first test. The samples are ordered according to relative number of days between tests. Only genes that were assayed in both ctDNA and tissue NGS tests are included on the Venn diagrams. For patients with serial ctDNA tests, all unique alterations over the serial testing dates were included once in the diagram.

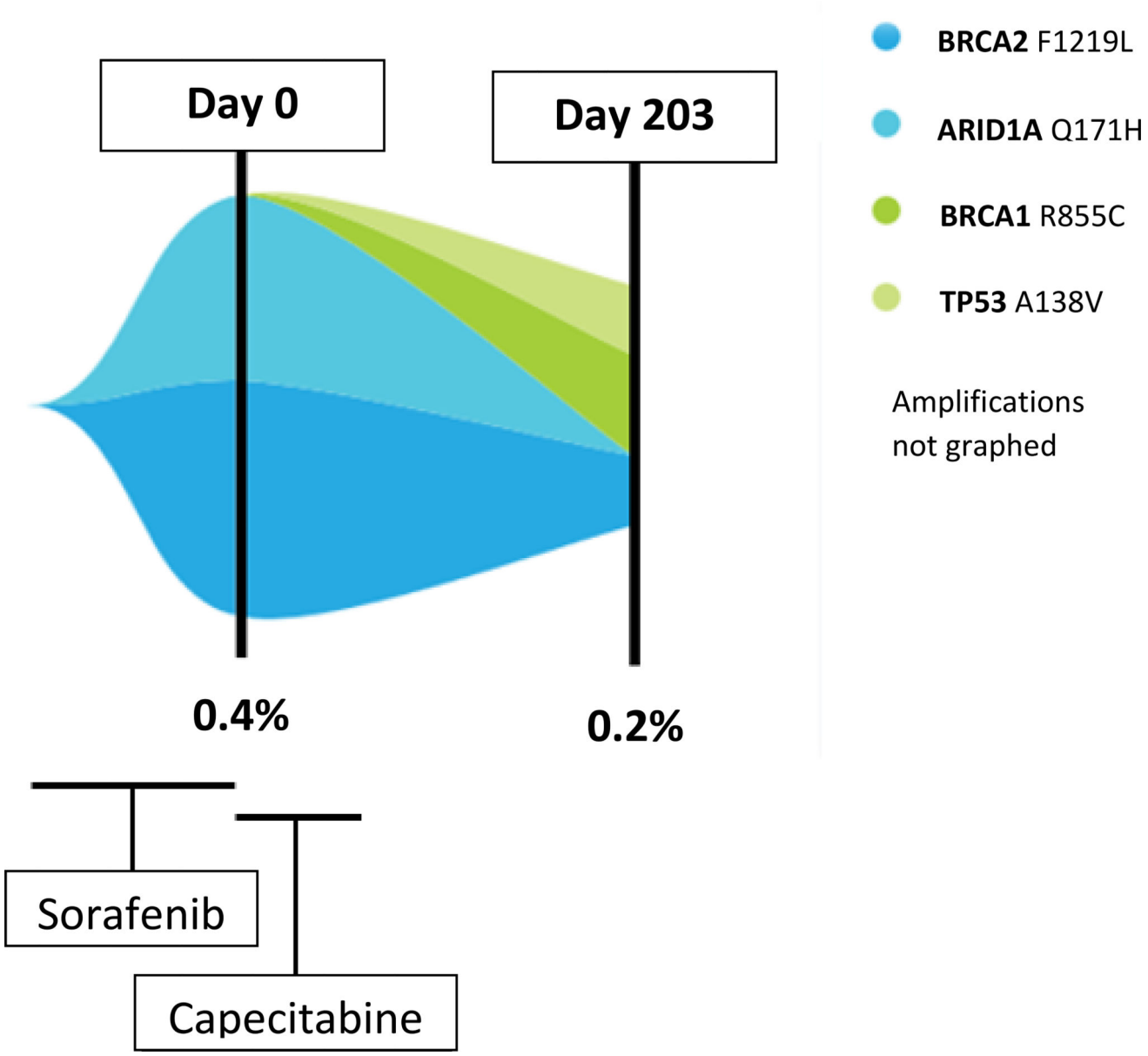

Figure 3. Serial analysis of ctDNA (Table 2, patient #10)

This figure shows the serial analysis of one patient over 203 days. Two serial ctDNA biopsies were conducted. The patient had been treated with sorafenib, but disease had shown progression. The patient was then treated with capecitabine starting on Day 0, which represents the date of the first liquid biopsy. The patient came off therapy because of side effects and progression. Liquid biopsy later shows a decrease in the original ctDNA alterations but emergence of new alterations. The X-axis represents time. The Y-axis represents the fraction of mutant allele frequency of each genomic alterations. The *ARID1A* and *BRCA2* ctDNA alterations (both VUSs) decreased but *BRCA1* (VUS) and *TP53* alterations emerged.

Table 1

Demographic and baseline characteristics of 26 patients with HCC

Variable	N=26
Median age - year	65 (range 44–74)
Sex – no. (%)	
Men	20 (76.9%)
*BCLC stage – no. (%)	
B	3 (11.5%)
C	12 (46.2%)
D	3 (11.5%)
*Child-Pugh class – no. (%)	
A	4 (15.4%)
B	13 (50.0%)
C	7 (26.9%)
N/A	2 (7.7%)
ECOG performance status – no. (%)	
0	2 (7.7%)
1	8 (30.8%)
2	11 (42.3%)
3	5 (19.2%)
Risk Factors: All patients – no. (%)	
Hepatitis B	1 (3.8%)
Hepatitis C	17 (65.4%)
Alcohol	3 (11.5%)
Metabolic Syndrome	5 (19.2%)
Number of patients with tissue next generation sequencing	10 (38.4%)
Risk Factors: Ten patients with both liquid and tissue biopsy – no. (%)	**N=10**
Hepatitis B	0 (0.0%)
Hepatitis C	8 (80.0%)
Alcohol	1 (10.0%)
Metabolic Syndrome	1 (10.0%)
Number of patients with ≥ 1 characterized alteration (%)	23 (88.5%)

Abbreviations: BCLC = Barcelona Clinic Liver Cancer Staging; ECOG = Eastern Cooperative Oncology Group; HCC = hepatocellular carcinoma

*Data not available for all patients

Table 2

Risk factors, genomic alteration pattern, and percent ctDNA in 26 patients with HCC

Patient	Risk Factor	Tissue NGS/Molecular**	Relative Date of Tissue NGS	Relative Date of Serial ctDNA	ctDNA Characterized Alterations (%ctDNA)	ctDNA VUS (%ctDNA)	AFP Levels (ng/mL) (Normal range, <15ng/ml)***	Child-Pugh Score (Class)
1	HBV	N/A	N/A	Day 0 *	TP53 R249S 33.31%		>60500.0	11 (C)
2	HCV	N/A	N/A	Day 0	CTNNB1 D32N 0.22%	CTNNB1 S29T 0.23% EGFR D837Y 0.20%	870	11 (C)
3	HCV	TP53 H179P ARID1A A226fs*5	Day 0	Day 828	TP53 H179P 37.77% BRAF amplification FGFR1 amplification CDK6 amplification EGFR amplification	ARID1A A226P 1.11%	**N/A**	N/A
4	HCV	TP53 R273C	Day 0	Day 460	MET Y501C 2.05% PTEN L139* 0.21% TP53 R273C 1.18%		13480	11 (C)
				Day 592	PTEN L139* 0.65% TP53 R273C 1.01% TP53 H179Y 0.18% EGFR E20fs 0.06%			
				Day 624	PTEN L139* 0.15% TP53 R273C 1.63% TP53 H179Y 0.13%			
5	HCV	N/A	N/A	Day 0	TP53 R175H 29.51% CTNNB1 S33C 13.02%	ARID1A Q2207R 0.55%	374000	11 (C)
6	HCV	N/A	N/A	Day 0	CDKN2A R80* 0.55% CTNNB1 G34V 1.35%		10	9 (B)

Patient	Risk Factor	Tissue NGS/Molecular**	Relative Date of Tissue NGS	Relative Date of Serial ctDNA	ctDNA Characterized Alterations (%ctDNA)	ctDNA VUS (%ctDNA)	AFP Levels (ng/mL) (Normal range, <15ng/ml)***	Child-Pugh Score (Class)
7	Metabolic Syndrome	AKT2 amplification CCNE1 amplification TP53 D281E	Day 0	Day 91	CCNE1 amplification CDK6 amplification PIK3CA amplification EGFR amplification MYC amplification		448100	6 (A)
8	Metabolic Syndrome	N/A	N/A	Day 0	CTNNB1 H36P 12.09% RAF1 I634F 0.12% TP53 H179R 0.13% RAF1 amplification	MAP2K1 V60M 0.67% ATM R337C 0.27% ALK R1347Q 0.12%	3	8 (B)
				Day 156	CTNNB1 H36P 31.54% TP53 R273H 0.18% TP53 H179R 0.12% RAF1 amplification	MAP2K1 V60M 0.35%		
9	HCV	N/A	N/A	Day 0	TP53 H193R 0.92%	APC N1919K 0.26% ERBB2 V1128I 8.71%	13	6 (A)
10	HCV	RB1 loss exons 7–17 TERT promoter −124C>T	Day 0	Day 431		ARID1A Q171H 0.31% BRCA2 F1219L 0.42%	7360	7 (B)
				Day 634	TP53 A138V 0.12%	BRCA1 R866C 0.19% BRCA2 F1219L 0.12%		
11	Alcohol	N/A	N/A	Day 0	TP53 R273C 12.08% ERBB2 amplification MYC amplification	APC T683I 2.74%	8990	10 (C)
				Day 133	TP53 R273C 40.57% ERBB2 amplification MYC amplification	APC T683I 10.09%		

Patient	Risk Factor	Tissue NGS/Molecular**	Relative Date of Tissue NGS	Relative Date of Serial ctDNA	ctDNA Characterized Alterations (%ctDNA)	ctDNA VUS (%ctDNA)	AFP Levels (ng/mL) (Normal range, <15ng/ml)***	Child-Pugh Score (Class)
12	Metabolic Syndrome	N/A	N/A	Day 0	TP53 S241F 0.13%	ESR1 P592R 0.10%	4	9 (B)
13	Alcohol	N/A	N/A	Day 0	ARID1A Q268* 0.21%	APC S2533F 0.10% ARID1A E1531K 0.27% RAF1 E278K 0.23% SMAD4 V354E 1.77%	72	7 (B)
14	Alcohol	CCND1 amplification ERRFI1 S302fs*10 VEGFA amplification FGF19 amplification	Day 0	Day 34		NFE2L2 F37S 0.48% FGFR2 G570S 0.12% CCND1 E135K 0.10%	75	8 (B)
15	HCV	N/A	N/A	Day 0		CDK6 H297Y 0.27% MYC R98W 0.30%	N/A	N/A
16	HCV	No alterations	Day 0	Day 876		ARID1A E2078K 0.12%	1.9	7 (B)
17	HCV	CTNNB1 S45P GNAS R201C TP53 W146fs*2 IRF2 W46* TERT promoter −124C>T	Day 0	Day 308 Day 350	CTNNB1 S45P 15.35% TP53 H214R 0.29% CTNNB1 S45P 26.04% TP53 H214R 0.39%	CCND2 D264G 1.26% NF1 G2499S 0.19% FGFR2 K539E 0.13% CCND2 D264G 1.58% RET K710N 0.17%	5	10 (C)
18	HCV	N/A	N/A	Day 0	ERBB2 R143Q 0.12% TP53 H178D 2.27%	CDKN2A S12L 0.38% KIT D975N 0.11% NF1 R2179C 0.10%	1256	8 (B)
19	HCV	CDKN2A p16INK4a M52fs*1 p14ARF H66fs*68+ CTNNB1 S45F KEAP1 R260*	Day 0	Day 671	CTNNB1 S45F 2.85%		1899	8 (B)

Patient	Risk Factor	Tissue NGS/Molecular**	Relative Date of Tissue NGS	Relative Date of Serial ctDNA	ctDNA Characterized Alterations (%ctDNA)	ctDNA VUS (%ctDNA)	AFP Levels (ng/mL) (Normal range, <15ng/ml)***	Child-Pugh Score (Class)
		RBM10 Q518 * TERT promoter −124C>T						
20	Metabolic Syndrome	N/A	N/A	Day 0	TP53 K139N 0.69%	ATM R337C 0.43% JAK3 M576K 0.44%	2134	7 (B)
21	HCV	FBXW7 E113D – subclonal CDKN2A/B loss AXIN1 R395P – subclonal TERT promoter −124C>T	Day 0	Day 63	PIK3CA H1047L 0.55% TP53 R280G 2.90% TP53 H193R 2.57%	KRAS I24L 2.29%	55	7 (B)
22	Metabolic Syndrome	N/A	N/A	Day 0	TP53 C176F 7.95%	MET P4S 6.48%	2	6 (A)
23	HCV	KIT V399I ARID1A E1668 * CTNNB1 S37F TERT promoter −124C>T	Day 29	Day 0	ARID1A E1668* 0.53% CTNNB1 S37F 0.75%	ATM N3003S 0.35% BRCA1 N941D 1.35%	17.2	6 (A)
24	HCV	N/A	N/A	Day 0	TP53 R249S 31.96% TP53 R196Q 0.19% KRAS amplification CCNE1 amplification CDK4 amplification	FGFR1 E464K 0.20%	571000	11 (C)
25	HCV	N/A	N/A	Day 0	MET I491T 0.14%		2	8 (B)
26	HCV	N/A	N/A	Day 0	AR R608Q 0.68% CTNNB1 T41A 2.35% NFE2L2 E82G 1.91% TP53 R248G 2.03%	KIT D975N 0.27% MYC S154L 0.18% NTRK1 E413K 0.15% RB1 M704R 1.69% TSC1 E1101K 0.11%	3	7 (B)

Abbreviations: ctDNA = circulating tumor DNA; HBV = hepatitis B; HCC = hepatocellular carcinoma; HCV = hepatitis C; N/A = not available; VUS = variant of unknown significance

*Day 0 represents the original test date.

**All patients had genomic testing by Foundation One (http://foundationone.com/), except for patient #16, whose testing was performed by Molecular Health (626 genes)

***AFP level closest in date to ctDNA blood draw was chosen

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Table 3

Potential actionability: Examples of possible targeted therapies for specific characterized genomic alterations

Genomic Aberration	Examples of possible treatments
ARID1A	*ARID1A* is possibly targetable with EZH2 inhibitor (EPZ-6438*, NCT01897571) through a synthetic lethal mechanism (23).
BRAF	*BRAF* is targetable by *BRAF* inhibitors such as vemurafenib and dabrafenib (24).
CCNE1	Synthetic lethal screen showed CCNE1 amplified cells required ubiquitin pathway and was sensitive to proteasome inhibitor bortezomib. CCNE1 mutations may be actionable with bortezomib, a proteasome inhibitor (25).
CDK4	*CDK4* may be targetable by *CDK4/6* inhibitors such as palbociclib (26).
CDK6	Palbociclib is a *CDK4/6* inhibitor (26).
CDKN2A	*CDKN2A* genomic alterations result in increased *CDK4/6* and are theoretically actionable with palbociclib, a *CDK4/6* inhibitor (27).
CTNNB1	In preclinical study using hepatocellular carcinoma cell lines, sorafenib was capable of inhibiting Wnt/β- catenin signaling (28). Sulindac (NSAIDs) also targets this pathway (29). *CTNNB1* may also be targeted by gamma secretase inhibitors; these inhibitors target Notch(30).
EGFR	*EGFR* amplification is targetable with cetuximab, an anti-*EGFR* therapy (31). *EGFR* alterations are actionable with erlotinib (31).
ERBB2	*ERBB2* alterations are actionable with lapatinib and trastuzumab (32).
FGFR1	*FGFR1* aberrations are potentially targetable with lenvatinib (33).
KRAS	*KRAS* mutations may be actionable with trametinib and other MEK inhibitors (34).
MET	*MET* is targetable by cabozantinib (35).
MYC	*MYC* can increase *CDK4* levels and thus *MYC* is potentially targetable with the *CDK4/6* inhibitor palbociclib (36). BET inhibitors downregulate *MYC* transcription (NCT01943851)(37).
PIK3CA	*PIK3CA* mutations are actionable with everolimus and other mTOR inhibitor (38).
PTEN	*PTEN* mutations are actionable with everolimus, a mTOR inhibitor (39).
TP53	*TP53* genomic alterations correlate with increased VEGF-A expression (40). A retrospective study suggests that patients with *TP53* mutations had longer progression-free survival with bevacizumab-containing therapies when compared to non-bevacizumab containing regimen (median 11.0 versus 4.0 months [$p < 0.0001$]) (41). Another report indicates that *TP53* mutations are associated with improvement in all outcome parameters when using VEGF/VEGFR inhibitors but that improvement is not seen in *TP53* wild-type patients (42). Finally, *TP53* mutations have been associated with better outcomes in sarcoma patients treated with the VEGFR inhibitor pazopanib (43). *TP53* may also be targetable by WEE1 inhibitors (44).

Abbreviations: NSAID = nonsteroidal anti-inflammatory drug