Original Article

Circulating tumor DNA correlates with microvascular invasion and predicts tumor recurrence of hepatocellular carcinoma

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Background: To evaluate the feasibility of predicting tumor recurrence of hepatocellular carcinoma (HCC) patients after curative hepatectomy by detection of circulating tumor DNA (ctDNA) through droplet digital PCR (ddPCR).

Methods: HCC patients receiving surgical treatment were enrolled and peripheral blood samples before and after hepatectomy were collected. Four hotspot mutants, *TP53*-rs28934571 (c.747G>T), *TRET*-rs1242535815 (c.1-124C>T), *CTNNB1*-rs121913412 (c.121A>G) and *CTNNB1*-rs121913407 (c.133T>C) were selected to detect ctDNA and the mutant allele frequency (MAF) was calculated accordingly. The matched peripheral blood mononuclear cells (PBMCs) were used for Sanger sequencing. The clinicopathologic information of the patients was retrospectively analyzed and the predictive abilities for postoperative recurrence of different clinicopathologic parameters and ctDNA were compared.

Results: Eighty-one patients were enrolled and 70.4% (57/81) of them had detectable ctDNA before hepatectomy. Positive preoperative ctDNA status was related to larger tumor size (P=0.001), multiple tumor lesions (P=0.001), microvascular invasion (MVI) (P<0.001), advanced BCLC stages (P<0.001) and shorter disease free survival (DFS) (P<<0.001) and overall survival (OS) (P<<0.001). Multivariate analysis showed that detectable ctDNA was the independent risk factor for postoperative recurrence. Moreover, receiver operating characteristic (ROC) curves proved that ctDNA possessed the second largest area under the curve (AUC) in foretelling postoperative recurrence right after BCLC stage. For patients after surgery, the alterations of MAF were also correlated to postsurgical recurrence. Patients with increased MAF had more incidences of MVI (P=0.016) and recurrence (P<0.001). At the same time, Kaplan-Meier curves revealed a significant shorter DFS and OS in the patients with increased MAF compared to the patients with decreased MAF (P<0.001 and P=0.0045, respectively) and ROC curves showed MAF to possess the greatest AUC among all the indices for postoperative recurrence.

Conclusions: Digital droplets PCR assessment of specific gene combination through ctDNA possesses potential prognostic value in HCC patients undergoing surgical treatment.

Keywords: Hepatocellular carcinoma (HCC); droplet digital PCR (ddPCR); postoperative recurrence; circulating tumor DNA (ctDNA); microvascular invasion (MVI)

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Introduction

Hepatocellular carcinoma (HCC) remains a great threat to public health worldwide (1). Surgery is the mainstream modality that can offer a cure for HCC patients. However, postoperative tumor recurrence happens in more than 60% HCC patients within 5 years (2) and stands as the main obstacle for further prolonging the overall survival (OS) (3). Thus, it's critical for clinical surgeons to predict patients with a high possibility of recurrence and apply suitable adjuvant therapy to improve the prognosis. Unfortunately, the current-existing clinical indices are hardly satisfactory in predicting tumor relapse. Being the most representative tumor marker, AFP has been serving to diagnose HCC and detect postoperative recurrence for decades, yet its sensitivity and specificity are still to be improved (4-6). Other markers such as PIVKA-II, AFP-L3 and Glypican-3 (GPC3) were also proved to be useful in HCC detection and surveillance (7,8), but they still served as complementary modalities to AFP (6). More importantly, the diagnostic value for recurrence of these markers was mostly effectuated by monitoring the fluctuating patterns (9) which called for continuous medical tests and was unlikely to be completed within perioperative periods. Therefore, novel method that can timely predict postoperative tumor recurrence is urgently needed.

In recent years, liquid biopsy has been greatly employed in the oncology research as a non-invasive and highly-sensitive technology (10). Specially, circulating free DNA or cell free DNA (cfDNA) has gained researchers' interest and been used in the field of tumor prognosis, diagnosis and treatment choice (10-12). As an unneglected part of cfDNA and originated from tumor cells (13), circulating tumor DNA (ctDNA) has also been brought to clinical usage. It's noteworthy that, ctDNA has been reported to predict tumor recurrence months ahead of image evidence (14), thus providing more chance for clinical intervention and prolonging OS. By far, the prognostic value of cfDNA or ctDNA was executed through concentration (15-19), which might be influenced by the extraction methods and patients' physiological states (20-24). Previously, we have reported that ctDNA could be detected in HCC patients using hotspot mutants on the droplet digital PCR (ddPCR) platform. However, it's still unknown whether ctDNA could predict tumor recurrence in HCC. Herein, for the first time, we systematically assayed the pre- and postoperative ctDNA in HCC patients using this ddPCR platform and found that positive ctDNA mutants correlated with microvascular invasion (MVI) and predicted tumor recurrence.

Methods

Patients' enrollment

From March 2013 to July 2015, patients hospitalized in Zhongshan Hospital, Fudan University were enrolled based on the following criterion: (I) no previous history of malignancy; (II) no synchronous malignancies in other organs; (III) no anti-tumor treatments before hepatectomy; (IV) curative hepatectomy; (V) the pathological diagnosis of the tumor was HCC.

Sample collection and preparation

Ten milliliter EDTA tube (BD, Plymouth, UK) was used to collect blood drawn from ulnar vein. Blood samples were obtained in the morning following hospitalization and on the 7th postoperative day, respectively. A two-step centrifugation method was adopted to process the blood samples within 3 hours: (I) 10 minutes' spin at 3,000 rpm to separate the blood cells; (II) another 10 minutes' spin at 13,000 rpm for the removal of the cellular debris. The clarified plasma and PBMCs were stored at −80 ℃ for future ctDNA extraction. PBMCs were obtained from the blood cells centrifugated in the first step. Detailed methods of PBMCs and ctDNA acquiring were formulated in a previous research (25).

Sanger sequencing

DNA extracted from PBMCs was defined as germline DNA. Sequencing was performed after the germline DNA was amplified and purified. The processed DNA product was used in each reaction. The sequencing was carried out in parallel to ddPCR in order to avoid any cross-interference.

ddPCR

The ddPCR was carried out on the QX200 platform (Bio-Rad, Hercules, USA) according to the instructions. Firstly,

10 μL 2× ddPCR SuperMix (Bio-Rad), 1 μL forward and reverse primer (1:1 mixture), 1 μL probe for wild and mutant type (1:1 mixture), 3 μL deionized distilled water and 5 μL ctDNA template were blended into a 20 μL volume system. Secondly, the reaction system was jointly loaded to droplet generator together with 70 μL droplet generation oil to form 13,000 to 20,000 droplets. Then the droplets were transferred into a 96-well PCR plate in aliquots of 40 μL for amplification. The sequence information was presented in *Table S1*.

The PCR plate was placed in the Droplet Reader (Bio-Rad) after the amplification and was analyzed by QuantaSoft (Bio-Rad). Allele concentrations of mutant (C_{MUT}, copies/μL) and wild type (C_{WT}, copies/μL) were calculated and mutant allele frequency (MAF) was defined as $C_{MUT}/(C_{MUT} + C_{WT})$, detailed PCR programmes and MAF calculation were described in a previous study (25).

Follow-ups

Follow-up evaluations were performed 1 month after surgery, then every 3 months within the first two years, every 6 months since the third postoperative year. Abdominal ultrasonography and levels of serum tumor markers (such as AFP) were regularly examined in every follow-up. Abdominal computed tomography (CT) or magnetic resonance imaging (MRI) was commenced if necessary. Any suspicious recurrence was confirmed or ruled out by an immediate abdominal enhanced MRI, chest CT or positron emission tomography (PET)/CT. Puncture biopsy was performed when the imaging diagnose was ambiguous. All patients were followed regularly till recurrence, death, or termination of the study. Disease free survival (DFS) and OS were used to represent the interval from the surgery to recurrence and from the surgery to death, respectively.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 (IBM). Clinical parameters in different groups were compared using Chi-Square test. Cox regression model was established for univariate and multivariate analyses. The area under the curve (AUC) of receiver operating characteristic (ROC) curve was calculated to evaluate the diagnostic utility of ctDNA and MAF. The DFS and OS were compared by Kaplan-Meier curves. All P values were two-sided and $P<0.05$ was considered statistically significant.

Results

Patients' demographics

Totally, 81 patients diagnosed with HCC were enrolled. The clinicopathological and epidemiological information were summarized in *Table 1*. The majority of the patients were male (69/81, 85.2%) with a median age of 54.7 (range, 28–78) years old. In all, 69 patients were HBsAg positive and 68 patients had liver cirrhosis. Before surgery, 46 patients had AFP level above 20 ng/mL, 61 patients' Child-Pugh Scores were rated A and 43 patients were classified as BCLC (Barcelona Clinic Liver Cancer) stage (26) 0 or A while 38 patients were classified as B to C. The patients' distributions according to the guidelines for primary liver cancer in China (27) were Ia [39], Ib [23], IIa [14] and IIb [5]. All patients successfully received hepatectomy with curative intent, and the Edmonson grades of their tumors were I–II [56] and III–IV [25].

Determination of lower of detection (LOD) of ddPCR platform and baseline ctDNA status

In our pilot research, we have established the LOD of QX200 platform by using serial dilution of *KRAS* G12D mutant (25). However, this mutant was not a frequent event in HCC. Herein, we further confirmed the platform's working performance by additionally evaluating the LOD using the hotspot mutant *TP53* rs28934571 of HCC. We compared the preset MAF with the measured MAF by ddPCR. As shown in *Figure S1*, the platform could stably detect the mutant allele at the MAF of 5%, 1%, 0.1% and 0.01% respectively, which was consistent with our previous result. Thus, the LOD in our study was 0.01%.

We then detected the four mutant alleles in the preoperative plasma DNA using this ddPCR platform. Totally, 57 patients (70.4%) were found to be ctDNA positive before surgery, of which 45 patients had 1 positive mutant allele, 11 patients had 2 positive alleles and 1 patient had 3 mutants. In general, the MAFs ranged from 0.02% to 43.28%, which were all above the LOD, thus ruling out the possibilities of false positivity. *TP53* (c.747G>T) mutant was detected in 12 patients, *TERT* (c.1-124C>T) was found positive in 40 patients, and *CTNNB1* (c.121A>G)

Table 1 Clinical characteristics of enrolled patients

Clinical characteristics	Number of patients
Age, years	
≥60	27
<60	54
Gender	
Male	69
Female	12
HBsAg	
Negative	12
Positive	69
Cirrhosis	
No	13
Yes	68
Tumor size	
<5 cm	28
≥5 cm	53
Tumor number	
Single	45
Multiple	36
MVI	
No	27
Yes	54
Tumor encapsulation	
No	34
Yes	47
Edmonson grade	
I + II	56
III + IV	25
AFP	
<20 ng/mL	35
≥20 ng/mL	46
ALT	
<50 μ/L	64
≥50 μ/L	17
AST	
<40 μ/L	41
≥40 μ/L	40
Child-Pugh score	
A	61
B	20
BCLC stage	
0 + A	43
B + C	38

ALT, alanine aminotransferase; AST, aspartate transaminase.

and (c.133T>C) were presented in 12 and 8 patients, respectively. Also, we assayed the matched PBMCs for the corresponding hotspot mutations using sanger sequencing in order to exclude the possibility of germline mutation. In all the 81 patients, none of the mutants were detected in the germline DNA. Thus, the mutants detected in plasma DNA were genuinely ctDNA. Detailed mutation information of ddPCR was presented in *Table S2*.

Preoperative ctDNA status correlates with prognosis

We then tried to analyze whether there was a correlation between preoperative ctDNA status and clinicopathological characteristics, and found that positive plasmatic ctDNA status before surgery significantly correlated with larger tumor size (P=0.001), more tumor lesions (P=0.001), more incidences of MVI (P<0.001), more advanced BCLC stages (P<0.001) and more events of tumor recurrence (P=0.018). Other parameters, such as cirrhosis, etiology, patient's age did not significantly affect the detection rate of ctDNA (*Table 2*).

Then, we compared the DFS and OS between the ctDNA positive and negative groups using Kaplan-Meier curves. As shown in *Figure 1*, positive preoperative ctDNA status was associated with both shorter OS (mean OS 22.5 *vs.* 40.0 months, P<0.001, *Figure 1A*) and DFS (mean DFS 16.6 *vs.* 35.3 months, P<0.001, *Figure 1B*).

Next, cox regression models were established and the univariate and multivariate analyses were carried out to reveal the risk factors for tumor recurrence of the patients from the common clinical markers. We found that ctDNA status, high AFP level (AFP ≥400 ng/mL), ALT level and BCLC stage were the risk factors for postsurgical recurrence in univariate analysis. And ctDNA, ALT and BCLC stage were further proved to be the risk factors for recurrence by multivariate assessment (*Table 3*).

On the bias of the multivariate analysis, we generated ROC curves to evaluate the ability of each risk factor to predict postoperative tumor relapse in HCC patients (*Figure 1E*). The AUCs, 95% confident interval (CI) and P values were summarized in *Table 4*. Following BCLC stage, ctDNA ranked the second place in predicting postoperative recurrence among all the parameters assessed, with very close AUCs (0.625 *vs.* 0.675).

ctDNA dynamic changes predict tumor recurrence

The postoperative plasma samples of 53 preoperative ctDNA positive patients were available and we then

Table 2 Comparison of the clinical characteristics between preoperative ctDNA positive and negative group

Clinicopathologic parameters	Positive group (n=57)		Negative group (n=24)		P value*
	N	%	N	%	
Age, years					0.302
≥60	17	21.0	10	12.3	
<60	40	49.4	14	17.3	
Gender					0.322
Female	7	8.6	5	6.2	
Male	50	61.7	19	23.5	
HBsAg					0.322
Negative	7	8.6	5	6.2	
Positive	50	61.7	19	23.5	
Cirrhosis					0.447
No	8	9.9	5	6.2	
Yes	49	60.5	19	23.5	
Tumor size					0.001
<5 cm	13	16.0	15	18.5	
≥5 cm	44	54.3	9	11.1	
Tumor number					0.001
Single	25	30.9	20	24.7	
Multiple	32	39.5	4	4.9	
MVI					<0.001
No	10	12.3	17	21.0	
Yes	47	58.0	7	8.6	
Tumor encapsulation					0.596
No	25	30.9	9	11.1	
Yes	32	39.5	15	18.5	
Edmonson grade					0.775
I + II	40	49.4	16	19.8	
III + IV	17	21.0	8	9.9	
AFP					0.423
<20 ng/mL	23	28.4	12	14.8	
≥20 ng/mL	34	42.0	12	14.8	
ALT					0.224
<50 μ/L	43	53.1	21	25.9	
≥50 μ/L	14	17.3	3	3.7	
AST					0.943
<40 μ/L	29	35.8	12	14.8	
≥40 μ/L	28	34.6	12	14.8	
Child-pugh score					0.242
A	45	55.5	16	19.8	
B	12	14.8	8	9.9	
BCLC stage					<0.001
0 + A	23	28.4	20	24.7	
B + C	34	42.0	4	4.9	
Recurrence					0.018
No	24	29.6	17	21.0	
Yes	33	40.7	7	8.6	

*, analysis by two-sided Pearson's Chi-square test, with P<0.05 considered significant. ctDNA, circulating tumor DNA; ALT, alanine aminotransferase; AST, aspartate transaminase.

evaluated whether ctDNA still existed upon tumor resection. Considering intratumoral heterogeneity (ITH) may hinder comprehensive genomic profiling, we assayed all the four mutants rather than the positive allele before surgery. We found that ctDNA disappeared in 23 (23/53, 43.4%) patients and the MAF of ctDNA decreased in 13 (13/53, 24.5%) patients. In 17 (17/53, 32.1%) patients, the MAF of ctDNA increased or novel mutants that were not detectable before surgery were observed.

In order to evaluate the clinical significance of ctDNA dynamics, we classified the patients into two groups: the ones with negative mutant or decreased ctDNA MAFs were in decreased group while the others with novel mutant or increased ctDNA MAFs were in the increased group. The tumor features were compared between the two groups (*Table 5*). Patients with increased ctDNA MAF postoperatively had a markedly higher recurrence rate (P<0.001) and MVI positivity percentage (P=0.016) compared to those with decreased ctDNA MAF. Kaplan-Meier analysis showed that the mean OS was 16.8 months for the increased group and 25.3 months for the decreased group (P=0.0045, *Figure 1C*), while the DFS for the increased group was 7.0 nd 20.8 months for the decreased group (P<0.001, *Figure 1D*). We also performed cox

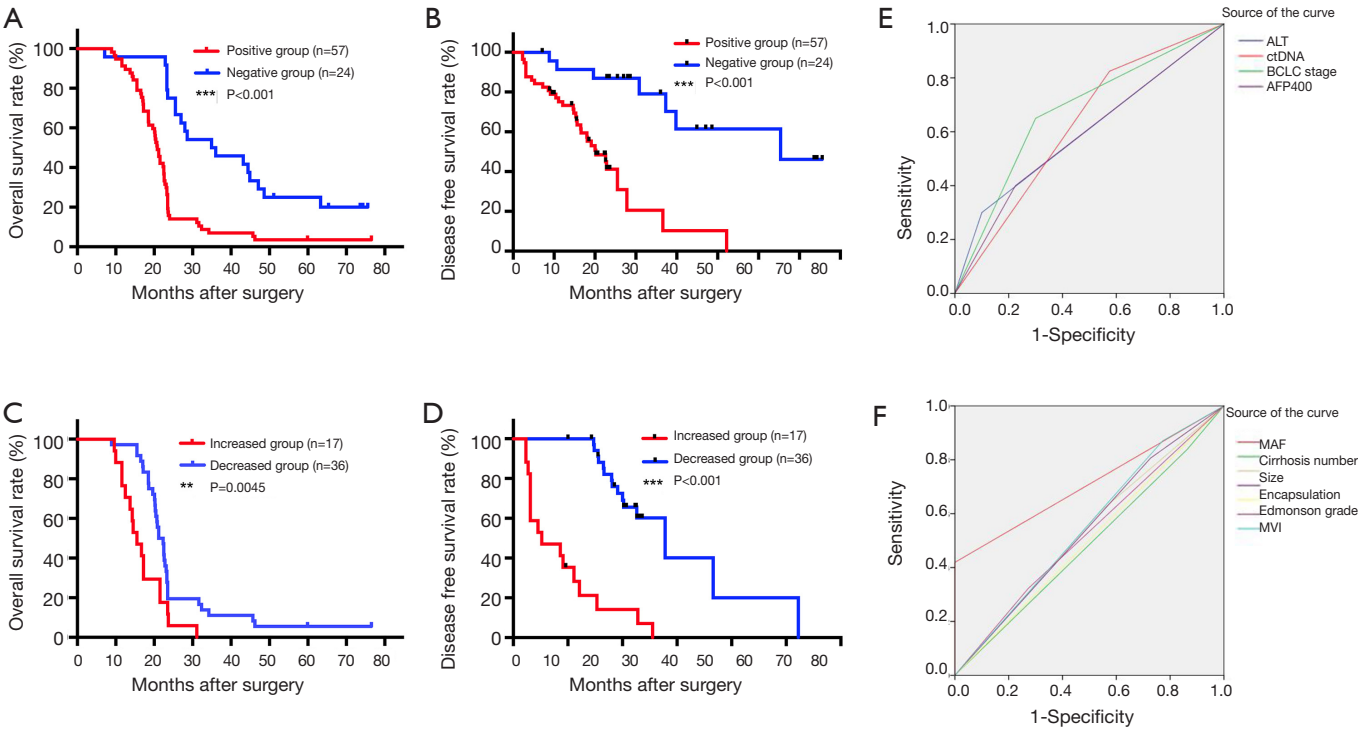

Figure 1 Comparison of OS, DFS between different groups and diagnostic ability for recurrence of various clinical indices. (A) Mean OS between ctDNA positive and negative patients was 22.5 *vs.* 40.0 months, P<0.001; (B) mean DFS between ctDNA positive and negative patients was 16.6 *vs.* 35.3 months, P<0.001; (C) mean OS between patients with increased and decreased MAF was 16.8 *vs.* 25.3 months, P=0.0045; (D) mean DFS between patients with increased and decreased MAF was 7.0 *vs.* 20.8 months, P<0.001; (E) preoperative ctDNA status ranked the second place in predicting postoperative recurrence among all the parameters assessed with an AUC of 0.625; (F) MAF possessed the greatest AUC (0.710) in predicting postoperative recurrence. OS, overall survival; DFS, disease free survival; ctDNA, circulating tumor DNA; AUC, area under the curve; MAF, mutant allele frequency.

regression analysis to reveal the correlated risk factors for postoperative recurrence in these patients. Both univariate and multivariate analyses showed that MVI and ctDNA MAF were the two independent risk factors for recurrence (*Table 6*) and ctDNA MAF possessed the greatest AUC for predicting postoperative recurrence in the ROC curves (*Table 7* and *Figure 1F*).

Discussion

HCC ranks the sixth in morbidity and the fourth in mortality worldwide (28) while in China the fifth in both morbidity and cancer-related deaths (29). Due to the endemic hepatitis B virus (HBV) infection, Chinese population accounted for more than half the new cases of HCC (30). The features of multistep and multicentric carcinogenesis of HCC often result in cancer recurrence and poor prognosis (31). Therefore, early prediction and detection of HCC recurrence are critical in prolonging patients' survival. However, even the most applied biomarker, AFP, with the cutoff value at 20 ng/mL, only had the sensitivity and specificity ranging from 41% to 65% and 80% to 94%, respectively (5). Therefore, novel biomarkers which could predict and detect HCC recurrence reliably and timely are urgently needed.

Recent years witnessed concentrated attentions on the studies of ctDNA. Harboring broad genetic information, ctDNA functions as a more comprehensive tool in analyzing tumoral genome compared to conventional sampling method (10). The relevant fields include unveiling drug resistance (11), monitoring treatment response (12), early diagnosis (32) and detection of incipient recurrence (14). Detailed research revealed close correlations between tumor recurrence and detectable postsurgical ctDNA as well as

Table 3 Univariate and multivariate analysis of preoperative indexes for recurrence by cox regression model

Clinicopathologic parameters	Univariate		Multivariate	
	HR (95% CI)	P value*	HR (95% CI)	P value*
Age, y: <60 *vs*. ≥60	0.521 (0.247–1.097)	0.086		
Gender: female *vs*. male	0.852 (0.302–2.406)	0.763		
ctDNA status: negative *vs*. positive	6.521 (2.520–16.874)	<0.001	4.204 (1.498–11.800)	0.006
AFP, ng/mL: <20 *vs*. ≥20	1.680 (0.880–3.205)	0.116		
AFP, ng/mL: <400 *vs*. ≥400	2.015 (1.062–3.823)	0.032	1.100 (0.530–2.284)	0.798
ALT μ/L; <50 *vs*. ≥50	2.392 (1.203–4.747)	0.013	2.238 (1.103–4.540)	0.026
AST μ/L; <40 *vs*. ≥40	1.381 (0.729–2.617)	0.322		
HBsAg: negative *vs*. positive	0.986 (0.410–2.370)	0.974		
INR: <1.2 *vs*. ≥1.2	1.182 (0.414–3.378)	0.755		
TB, μmol/L: <17.1 *vs*. ≥17.1	1.094 (0.458–2.613)	0.839		
ALB, g/L: <40 *vs*. ≥40	1.664 (0.891–3.107)	0.110		
Child-Pugh score: A *vs*. B	0.985 (0.467–2.078)	0.969		
BCLC stage: 0 + A *vs*. B + C	4.028 (2.040–7.955)	<0.001	2.266 (1.029–4.987)	0.042

*, analysis by cox regression, with P<0.05 considered significant. INR = $(PT_{test}/PT_{normal})ISI$. ctDNA, circulating tumor DNA; ALT, alanine aminotransferase; AST, aspartate transaminase; PT, prothrombin time; TB, total bilirubin; ALB, albumin.

Table 4 AUCs, 95% CI and P values of different clinical parameters in predicting recurrence for HCC patients after surgery

Factors	AUC	95% CI	P value
ctDNA	0.625	0.502–0.748	0.054
AFP400	0.588	0.462–0.713	0.178
ALT	0.600	0.475–0.725	0.124
BCLC stage	0.675	0.556–0.794	0.007

AUC, area under the curve; HCC, hepatocellular carcinoma; ctDNA, circulating tumor DNA; ALT, alanine aminotransferase.

dynamic ctDNA changes (33,34).

Being one of the commonly used modalities that can overcome the low fraction ctDNA accounts for in cfDNA (35), ddPCR allows the detection of mutant down to 0.001%, which is superior to qPCR (36). In our study, ctDNA extracted from less than 5 mL peripheral blood plasma was sufficient for ddPCR reactions, this also highlighted the low demand of sample capacity (36). At the same time, ddPCR also provides a possibility of absolute quantification of nucleic acids since each reaction takes place in an individual droplet. However, considering the limited throughput expected from ddPCR and multi-genetic variations in HCC, a pre-time selection of targeted mutants was recommended. According to our previous studies, *TP53*, *CTNNB1* and *TERT* were the most reported mutated genes in HCC (37-42). By furtherly testifying the relevance between mutant types and liver carcinogenesis, we narrowed the targeted mutants down to the up-mentioned four spots (25).

Our results showed that the preoperative mutant detection rate was 70.4%, while the positive rate of AFP was only 56.8%. By adding more relevant hotspots, the detection rate could be furtherly improved, amplifying the advantages of ctDNA. More importantly, detectable ctDNA before surgery was directly related to tumor size, tumor number, MVI and BCLC stage, which linked the mutants harbored in ctDNA with the tumor characteristics. This might be explained by the following reasons, since ctDNA is often considered fragments of tumor DNA in circulation, larger tumor size, more tumor lesions and vascular invasion by the tumor indicate higher possibilities of ctDNA release (43), which may result in more detections. The close connection between ctDNA and tumor features makes ctDNA an ideal potential tumor marker for recurrence. Both the ROC and OS/DFS assessments proved our speculation. Not only were the OS and DFS significantly shorter in the ctDNA positive group, the AUC of ctDNA status was also greater

Table 5 Comparison of clinical features between increased MAF group and decreased MAF group

Clinicopathologic parameters	Increased group (n=17)		Decreased group (n=36)		P value*
	N	%	N	%	
Tumor size					0.267
<5 cm	2	3.8	9	17.0	
≥5 cm	15	28.3	27	50.9	
Tumor number					0.158
Single	5	9.4	18	34.0	
Multiple	12	22.6	18	34.0	
MVI					0.016
No	0	0	10	18.9	
Yes	17	32.1	26	49.0	
Tumor encapsulation					0.335
No	9	17.0	14	26.4	
Yes	8	15.1	22	41.5	
Edmonson grade					0.066
I + II	9	17.0	28	52.8	
III + IV	8	15.1	8	15.1	
Recurrence					<0.001
No	1	1.9	21	39.6	
Yes	16	30.2	15	28.3	

*, analysis by two-sided Pearson's Chi-square test, with P<0.05 considered significant. MAF, mutant allele frequency.

Table 6 Univariate and multivariate analysis of MAF and other tumor features for recurrence by cox regression model

Clinicopathologic parameters	Univariate		Multivariate	
	HR (95% CI)	P value*	HR (95% CI)	P value*
Age, y: <60 *vs.* ≥60	0.588 (0.237–1.460)	0.252		
Gender: female *vs.* male	1.992 (0.572–6.943)	0.279		
ctDNA MAF: decreased *vs.* increased	16.827 (6.383–44.358)	<0.001	21.469 (7.497–61.480)	<0.001
Cirrhosis: no *vs.* yes	0.879 (0.334–2.317)	0.795		
Tumor size, cm: <5 *vs.* ≥5	2.215 (0.839–5.845)	0.108		
Tumor number: single *vs.* multiple	1.858 (0.874–3.951)	0.107		
MVI: no *vs.* yes	5.103 (1.183–22.008)	0.029	6.378 (1.449–28.070)	0.014
Tumor encapsulation: no *vs.* yes	0.664 (0.319–1.382)	0.274		
Edmonson grade: I + II *vs.* III + IV	1.344 (0.621–2.909)	0.453		

*, analysis by cox regression, with P<0.05 considered significant. MAF, mutant allele frequency; ctDNA, circulating tumor DNA; MVI, microvascular invasion.

Table 7 AUCs, 95% CI and P values of different clinical parameters in predicting outcome for HCC patients after surgery

Factors	AUC	95% CI	P value
ctDNA MAF	0.710	0.572–0.847	0.01
Cirrhosis	0.488	0.329–0.646	0.878
Number	0.534	0.375–0.693	0.678
Size	0.540	0.380–0.699	0.626
Encapsulation	0.501	0.342–0.661	0.986
Edmonson grade	0.525	0.366–0.683	0.759
MVI	0.549	0.389–0.709	0.545

AUC, area under the curve; HCC, hepatocellular carcinoma; ctDNA, circulating tumor DNA; MAF, mutant allele frequency; MVI, microvascular invasion.

than AFP. Though the diagnostic value of ctDNA was topped by BCLC stage, considering the limited mutation spots, the performance of a more comprehensive and well-established mutant panel for ctDNA is worth waiting.

It is reported that the half-life time of ctDNA in circulation was no more than 2 hours (44); thus it's reasonable to expect either a complete loss or a detectable decrease in MAF by the time we performed the second ddPCR and assume that the mutants detected in plasma 7 days after surgery were all from tumor cells still remaining in the liver, either as early intrahepatic metastasis or from the positive surgical margin. Apparently, disappearance of ctDNA or decrease of MAF was observed in most patients after resection, which should be the result of tumor removal. Still, we found increase or new-onset of mutant in some cases. We assume that this may be the result of the cancer biological behavior and ITH. Over half of the HCC patients still developed recurrence even with R0 resection and presence of minimally residual disease (MRD) was often considered to be responsible for that (45). These MRDs, being stimulated by surgical trauma, might function as the sources of the ctDNA after surgery, resulting in MAF increases. The close correlation between increased MAF and MVI furtherly proved our assumption. ITH played another key role in the MAF fluctuation. The vast amounts of mutants included within the HCCs laid the ground of great heterogeneity among the micro-lesions, causing possibilities of release of various kinds of hotspots from each focus, so that the ctDNA detected after surgery might harbor mutants different from the ones before surgery. Taken together, the detectable mutations in ctDNA after resection should be a solid evidence of MRD and indicate great chances of recurrence. This went along with our results. We found that patients with increased MAF manifested a significant shorter OS and DFS, moreover, the diagnostic value of MAF surpassed MVI, furtherly emphasizing the potential clinical application of ctDNA for recurrence.

Another interesting phenomenon was that we found that unlike the preoperative situation, the fluctuations of MAF and MVI were the only two factors remaining for recurrence, tumor size or tumor number failed to maintain the correlation. We speculated that this might be caused by the curative resections. Although larger tumor size and more tumor lesions might result in higher possibilities of recurrence (46), complete removal of the tumor could reduce the rate. By thorough radiographic examinations, both tumor size and tumor number can be acquainted with, therefore, specified resection strategies would be formulated to ensure a curative hepatectomy. On the other hand, MVI could only be diagnosed by postoperative pathology, there were no effective means to predict or handle MVI before hands. This reflected the superiority of MVI and ctDNA MAF in foretelling recurrence compared to the other tumor features.

According to our study, both the preoperative ctDNA status and fluctuations of ctDNA MAF performed well in predicting postoperative HCC relapse, still, there are some differences between the two indices. Taken more as a preoperative marker, ctDNA status could be used to predict tumor relapse after resection, its diagnostic ability was proved to be superior to that of AFP. The fluctuations of MAF, on the other hand, are more of a reflection of the surgery. Declined MAFs could be indicating a complete tumor resection while upgoing MAFs or novel mutant spots might be the sign of MRDs or micro-metastasis.

There are still some limitations in our study. First, this is a domestic research mainly concerning about Chinese population, the result should be furtherly validated in other group of people. Second, although we have observed associations between preoperative ctDNA positive status and several clinicopathological factors, it should be cautiously interpreted since we have only employed four mutants to detect ctDNA and this may result in false negativity, which would bias the association. To evaluate how ctDNA might be correlated with clinicopathological factors, deep sequencing of ctDNA with a panel of recurrently mutated genes in HCC would be more appropriate.

In conclusion, our study demonstrated a close correlation between plasma ctDNA and tumor features

in HCC patients. Both preoperative ctDNA and dynamic fluctuations of MAF in ctDNA could be useful clinical markers for tumor relapse after surgery.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for full data access, integrity of the data and the accuracy of the data analysis and ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (the Institutional Review Board of Zhongshan Hospital, Fudan University) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from every patient.

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Supplementary

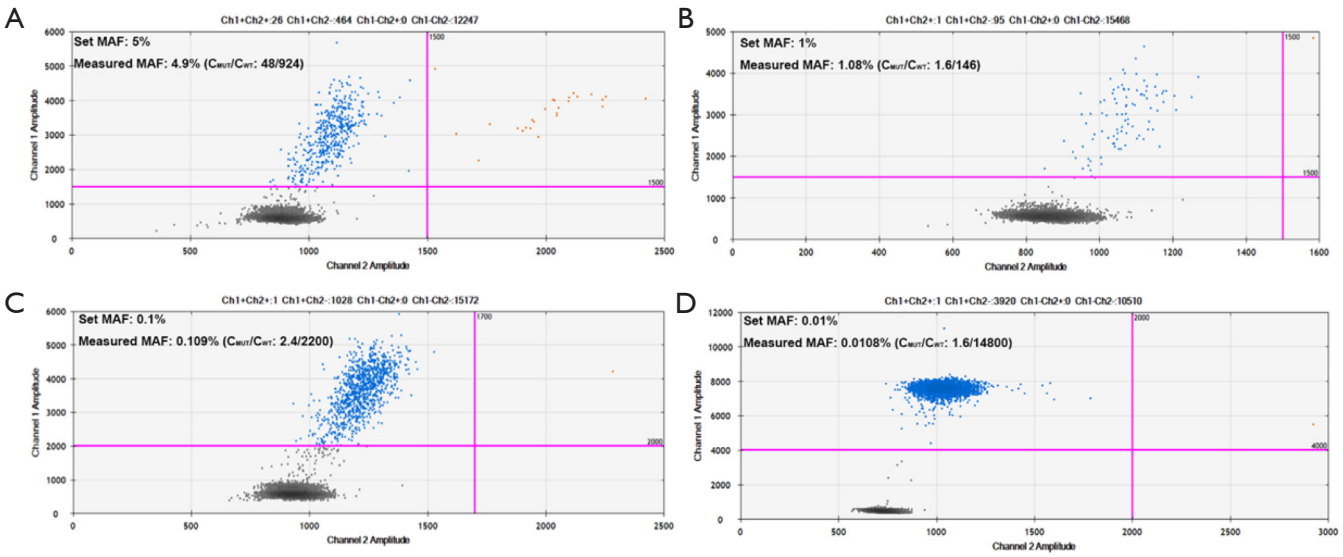

Figure S1 Verification of ddPCR accuracy by a mutant concentration gradient of *TP53* rs28934571. The results of ddPCR were in accordance with the pre-set MAF at 5% (A), 1% (B), 0.1% (C) and 0.01% (D). ddPCR, droplet digital PCR; MAF, mutant allele frequency.

Table S1 Sequence information of the primers and probes for the ddPCR assays

Mutation	Primer and probe	Sequence
TRET-rs1242535815	Forward primer	GAAAGGAAGGGGAGGGG
	Reverse primer	GCGCGGACCCCGCCCCGT
	Mutant probe	CCAGCCCCTTCCGGGCCCT
	Wild type probe	CCAGCCCCCTCCGGGCCCT
CTNNB1-rs121913412	Forward primer	GTTAGTCACTGGCAGCAACAGTCTTAC
	Reverse primer	GGGAGGTATCCACATCCTCTTCCTC
	Mutant probe	TGCCACTGCCACA
	Wild type probe	TGCCACTACCACA
CTNNB1-rs121913407	Forward primer	AGCAACAGTCTTACCTGGACTCTGG
	Reverse primer	CATACAGGACTTGGGAGGTATCCAC
	Mutant probe	AGCTCCTCCTCTG
	Wild type probe	AGCTCCTTCTCTG
TP53-rs28934571	Forward primer	CTGTACCACCATCCACTACAACT
	Reverse primer	AGCAGAGGCTGGGGCACAGCAGGC
	Mutant probe	ACCGGAGTCCCATCC
	Wild type probe	ACCGGAGGCCCATC

ddPCR, droplet digital PCR.

Table S2 Analysis of mutation status in plasma with ddPCR

Patients number	*TP53*-rs28934571			*CTNNB1*-rs121913407			*CTNNB1*-rs121913412			*TRET*-rs1242535815			Summary
	Preoperative MAF	Postoperative MAF	MAF change	Preoperative MAF	Postoperative MAF	MAF change	Preoperative MAF	Postoperative MAF	MAF change	Preoperative MAF	Postoperative MAF	MAF change	
HCC2	2.12%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	Decreased
HCC3	0.00%	0.00%	Na	0.23%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	Decreased
HCC4	0.24%	0.74%	Increased	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	Increased
HCC6	0.00%	0.00%	Na	0.13%	0.00%	Decreased	0.00%	0.00%	Na	1.30%	20.79%	Increased	Increased
HCC8	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.43%	0.18%	Decreased	Decreased
HCC9	0.00%	–	–	0.00%	–	–	1.61%	–	–	0.00%	–	–	–
HCC10	0.00%	0.00%	Na	1.96%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.09%	Increased	Increased
HCC12	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.25%	0.17%	Decreased	Decreased
HCC16	0.00%	0.00%	Na	0.00%	0.14%	Increased	0.00%	0.00%	Na	1.71%	0.00%	Decreased	Increased
HCC18	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.13%	0.00%	Decreased	Decreased
HCC19	0.08%	0.00%	Decreased	0.00%	0.10%	Increased	0.00%	0.00%	Na	0.28%	0.09%	Decreased	Increased
HCC21	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.14%	0.23%	Increased	Increased
HCC22	0.12%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.12%	Increased	Increased
HCC23	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.34%	0.09%	Decreased	Decreased
HCC25	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.46%	0.12%	Decreased	Decreased
HCC26	0.00%	0.00%	Na	1.17%	0.28%	Decreased	0.00%	0.00%	Na	0.57%	0.00%	Decreased	Decreased
HCC27	0.00%	0.00%	Na	0.02%	0.00%	Decreased	0.02%	0.00%	Decreased	0.06%	0.00%	Decreased	Decreased
HCC28	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	1.55%	0.41%	Decreased	Decreased
HCC29	0.00%	0.00%	Na	0.53%	0.00%	Decreased	0.00%	0.00%	Na	0.22%	0.00%	Decreased	Decreased
HCC30	0.00%	0.00%	Na	0.22%	0.13%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	Decreased
HCC33	0.00%	–	–	0.00%	–	–	0.13%	–	–	0.00%	–	–	–
HCC34	2.17%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	Decreased
HCC36	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.95%	0.00%	Decreased	Decreased
HCC37	0.00%	0.00%	Na	0.47%	0.00%	Decreased	0.00%	0.05%	Increased	22.95%	0.00%	Decreased	Increased
HCC38	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.42%	0.13%	Decreased	Decreased
HCC40	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	30.50%	0.22%	Decreased	Decreased
HCC42	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.68%	0.13%	Decreased	Decreased
HCC43	0.40%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	0.12%	0.32%	Increased	Increased
HCC44	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	1.54%	0.08%	Decreased	Decreased
HCC45	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.52%	0.00%	Decreased	Decreased
HCC46	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.23%	0.36%	Increased	Increased
HCC47	0.00%	0.26%	Increased	0.00%	0.00%	Na	0.00%	0.24%	Increased	32.37%	0.00%	Decreased	Increased
HCC48	0.18%	0.00%	Decreased	0.00%	0.08%	Increased	0.17%	0.08%	Decreased	0.28%	0.00%	Decreased	Increased
HCC49	1.77%	0.12%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	0.19%	0.00%	Decreased	Decreased
HCC50	0.00%	0.00%	Na	0.00%	0.11%	Increased	0.00%	0.05%	Increased	1.08%	0.00%	Decreased	Increased
HCC51	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.40%	0.00%	Decreased	Decreased
HCC53	0.00%	0.00%	Na	0.00%	0.00%	Na	0.90%	0.00%	Decreased	0.00%	0.00%	Na	Decreased
HCC55	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.15%	0.00%	Decreased	Decreased
HCC58	0.00%	0.00%	Na	0.00%	0.00%	Na	0.17%	0.09%	Decreased	0.00%	0.00%	Na	Decreased
HCC59	0.00%	0.00%	Na	0.07%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	Decreased
HCC60	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.27%	0.00%	Decreased	Decreased
HCC61	0.00%	0.00%	Na	0.56%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	Decreased
HCC62	0.00%	0.00%	Na	0.00%	0.00%	Na	0.12%	0.00%	Decreased	0.00%	0.00%	Na	Decreased
HCC63	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.27%	0.00%	Decreased	Decreased
HCC64	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	22.19%	0.00%	Decreased	Decreased
HCC65	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.39%	0.00%	Decreased	Decreased
HCC66	3.48%	0.00%	Decreased	0.00%	0.29%	Increased	0.00%	0.00%	Na	0.00%	0.00%	Na	Increased
HCC68	0.00%	0.00%	Na	1.19%	0.00%	Decreased	0.00%	0.00%	Na	6.71%	0.00%	Decreased	Decreased
HCC69	7.67%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	0.58%	0.00%	Decreased	Decreased
HCC70	0.00%	0.00%	Na	0.00%	0.18%	Increased	0.00%	0.00%	Na	1.27%	0.20%	Decreased	Increased
HCC72	0.00%	–	–	0.00%	–	–	0.09%	–	–	0.00%	–	–	–
HCC73	0.00%	0.00%	Na	0.00%	0.17%	Increased	0.00%	0.00%	Na	2.73%	0.17%	Decreased	Increased
HCC75	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.16%	0.00%	Decreased	Decreased
HCC76	0.00%	–	–	0.22%	–	–	0.00%	–	–	0.40%	–	–	–
HCC79	0.75%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.05%	Increased	4.94%	0.72%	Decreased	Increased
HCC80	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	0.44%	0.00%	Decreased	Decreased
HCC81	43.28%	0.00%	Decreased	0.00%	0.00%	Na	0.00%	0.00%	Na	0.00%	0.00%	Na	Decreased

Note: Na, MAF was both negative before and after surgery. ddPCR, droplet digital PCR; MAF, mutant allele frequency.