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Preoperative evaluation of microvascular invasion with circulating tumour DNA in operable hepatocellular carcinoma

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Abstract

Background & Aims: Microvascular invasion (MVI) is a critical prognostic factor for operable hepatocellular carcinoma (HCC). This study aimed to explore the performance of circulating tumour DNA (ctDNA) in evaluating MVI status preoperatively.

Methods: Seventy-three HCC patients were enrolled and randomly divided into a training cohort and a validation cohort in a 2:1 ratio, and preoperative blood and surgical tissue samples were obtained. Genomic alterations were analysed using targeted deep sequencing with a 1021-gene panel.

Results: In training cohort, 260 somatic mutations were identified in 40 blood samples (81.6%). CtDNA mutation was verified in paired tissue sample in 39 patients (97.5%). In univariate analysis, ctDNA allele frequency (AF) and largest tumour diameter were associated with the presence of MVI, but ctDNA AF was the only independent risk factor in multivariate analysis. With the cut-off value of 0.83%, ctDNA AF determined the presence of MVI with the sensitivity of 89.7% and specificity of 80.0% in the training cohort, and the sensitivity of 78.6% and the specificity of 81.8% in the validation cohort. In preoperative evaluation, ctDNA AF, AFP level and BCLC staging were associated with recurrence-free survival in both univariate and multivariate analysis.

Conclusions: CtDNA can serve as an independent risk factor of MVI for operable HCC and help determining precise treatment strategies. The integration of ctDNA in the management of operable HCC may achieve better clinical outcomes.

KEYWORDS

ctDNA, hepatectomy, hepatocellular carcinoma, microvascular invasion, surgical management

Abbreviations: AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; CA19-9, carbohydrate antigen 19-9; ctDNA, circulating tumour DNA; HBs-Ag, hepatitis B surface antigen; HCC, hepatocellular carcinoma; ICGR15, indocyanine green retention rate at 15 min; MELD, model for end-stage liver disease; MVI, microvascular invasion; PLT, platelet count; PT-INR, prothrombin time-international normalized ratio; RFS, recurrence-free survival.

1 | BACKGROUND

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death for male patients across developing countries, particularly in China.[1] Despite the rapid development of diverse therapeutic regimens, hepatectomy and liver transplantation are the main curative treatments.[2-4] The shortage of donors and strict liver transplantation criteria limit the application of liver transplantation, rendering hepatectomy as the first-line treatment for HCC.[5] The high incidence of recurrence, however, remains a major challenge in HCC therapy.[6,7]

Microvascular invasion (MVI), which is defined as over 50 malignant cells in the endothelial vascular lumen within a single cancer cell nest under microscopy,[8] has increasingly been recognized as an independent risk factor for recurrence and poor outcomes in HCC patients after hepatectomy,[6,8,9] and can help determining operation strategies (such as radical resection or not).[10,11] Currently, MVI can only be definitively identified via pathological examination. Other attempts about MVI prediction comprised imaging techniques, including compute tomography (CT), magnetic resonance imaging (MRI) and diffusion-weighted imaging (DWI), gene expression signatures, alpha fetoprotein (AFP) levels and other clinicopathologic characteristics, of which the prediction efficacy is still unsatisfying.[9,12-14] There is an urgent need to develop and improve the preoperative determination of MVI.

Circulating tumour DNA (ctDNA), which is mainly released by apoptotic or necrotic cancer cells, becomes a novel and noninvasive method for the diagnosis and monitoring of cancers and has increasingly been implemented in the experimental exploration of many cancers, including HCC.[15] Considering the possible coupling between MVI and ctDNA level, it may be a promising exploration to integrate ctDNA analysis into the preoperative prediction of MVI. Unknown is whether the predictive efficacy satisfies the clinical practice, as well optimal detection threshold need to be determined, which was primarily discussed in this study.

KEY POINTS

- Circulating tumour DNA can reliably reflect the genomic landscape, and serve as an independent risk factor of microvascular invasion for operable hepatocellular carcinoma.
- Our finding not only expands the potential applicability of circulating tumour DNA in clinical decision-making before surgery, but also enable the improvement of subsequent medical treatment in view that circulating tumour DNA profiling provide a promising way to reveal the molecular characteristics of cancer patients.
- Findings in this article also provided evidence for circulating tumour DNA as a biomarker in new clinical trials designed to determine precision medicine throughout the whole course of cancers.

2 | METHODS

2.1 | Patients

From December 2017 to June 2019, a total of 73 patients initially diagnosed with operable HCC at XiangYa Hospital affiliated to Central South University were enrolled in this study. All patients provided written informed consent. The study was approved by the Ethics Committee of Xiangya Hospital affiliated to Central South University (No. 201703377). These patients were then randomly divided into a training cohort and a validation cohort in a 2:1 ratio (finally 49 in the training cohort and 24 in the validation cohort). The general clinical characteristics of both cohorts are summarized in Table 1. Hepatectomy was conducted for all enrolled patients. After surgery, all patients were monitored regularly in the outpatient clinic with analysis of tumour markers, liver function tests and abdominal ultrasound. Further CT or MRI scans were performed if recurrence was suspected. Recurrence-free survival (RFS) was calculated from the date of liver resection to the date of diagnosis of tumour recurrence.

2.2 | Blood and HCC tissue sample collection

Peripheral blood samples were collected from each patient in EDTA Vacutainer tubes (BD Diagnostics) before hepatectomy. Fresh tumour specimens were obtained from patients according to a seven-point baseline sample collection protocol during surgery.[8] MVI was diagnosed postoperatively according to haematoxylin- and eosin-stained samples by two independents pathologists.[8]

2.3 | Sequencing library construction and target enrichment

DNA was extracted from the plasma and tumour tissues according to previously published methods.[16,17] Peripheral blood lymphocytes (PBLs) were used for the detection of germline mutations. Specifically, PBL DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen). Indexed Illumina next-generation sequencing libraries were prepared from tissue samples. Germline and ctDNA libraries were prepared using the KAPA Library Preparation Kit (Kapa Biosystems) as described previously.[17] Capture probe was designed to span the coding sequences or exon hotspots of the 1,021 genes frequently mutated in solid tumours (Table S1). Libraries were hybridized to custom-designed biotinylated oligonucleotide probes (Integrated DNA Technologies).

TABLE 1 Clinicopathological variables of patients

Variables	Analytical cohort (n = 49)
Age, years, mean (SD)	51.6 (13.4)
Gender	
Male, n (%)	46 (93.9%)
Female, n (%)	3 (6.1%)
HBs-Ag	
Negative, n (%)	4 (8.2%)
Positive, n (%)	45 (91.8%)
HBV-DNA	
≤10^4 IU/mL, n (%)	28 (57.1%)
>10^4 IU/mL, n (%)	21 (42.9%)
Child-Pugh grade	
A, n (%)	45 (91.8%)
B, n (%)	4 (8.2%)
MELD score, mean (SD)	5.6 (0.5)
ICGR15, %, mean (SD)	6.1 (6.1)
Albumin, g/L, mean (SD)	41.6 (5.1)
PLT, 10^9/L, mean (SD)	156.7 (63.3)
Total bilirubin, μmol/L, mean (SD)	12.7 (5.1)
Direct bilirubin, μmol/L, mean (SD)	6.2 (2.4)
PT-INR, mean (SD)	1.1 (0.1)
ALT, U/L, mean (SD)	39.8 (35.9)
AST, U/L, mean (SD)	43.4 (30.7)
AFP, ng/mL, mean (SD)	364.8 (445.0)
CA19-9, IU/mL, mean (SD)	22.7 (22.2)
Imaging results	
Tumour diameter, cm, mean (SD)	5.7 (3.5)
Tumour number	
Single, n (%)	39 (79.6%)
Multiple, n (%)	10 (20.4%)
Tumour capsule	
Complete, n (%)	7 (14.3%)
Incomplete, n (%)	42 (85.7%)
Cirrhosis	
Negative, n (%)	14 (28.6%)
Positive, n (%)	35 (71.4%)
BCLC staging	
0/A, n (%)	39 (79.6%)
B/C, n (%)	10 (20.4%)
Pathological results	
Edmondson-Steiner classification	
I-II, n (%)	37 (75.5%)
III-IV, n (%)	12 (24.5%)
MVI	
Negative, n (%)	20 (40.8%)
Positive, n (%)	29 (59.2%)

Abbreviations: HBs-Ag, hepatitis B surface antigen; MELD, model for end-stage liver disease; ICGR15, indocyanine green retention rate at 15 min; PLT, platelet count; PT-INR, prothrombin time-international normalized ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, alpha fetoprotein; CA19-9, carbohydrate antigen 19-9; MVI, microvascular invasion.

2.4 | Targeted deep sequencing and Variant Calling

Sequencing was performed with an Illumina HiSeq 3000 instrument with 2 × 75 bp paired-end reads according to the manufacturer's recommendations using the TruSeq PE Cluster Generation Kit v3 and the TruSeq SBS Kit v3 (Illumina). After removal of terminal adaptor sequences and low-quality reads (>50% N rate, >50% bases with Q <5), remaining reads were mapped to the reference human genome (hg19) and aligned using the Burrows-Wheel Aligner (version 0.7.12-r1039, http://bio-bwa.sourceforge.net/) with default parameters, followed by duplicate reads identification using the Picard's Mark Duplicates tool (https://software.broadinstitute.org/gatk/documentation/tooldocs/4.0.3.0/picard_sam_ markdplicates_MarkDuplicates.php). Base quality recalibration and local realignment were conducted by the Gene Analysis Toolkit (GATK, https://www.broadinstitute.org/gatk/). The median effective sequencing depth for ctDNA and tissue DNA were 2458× (ranged from 461 to 11 896×) and 739× (ranged from 207 to 1223×) respectively. Somatic insertions/deletions and single nucleotide variants were called using the MuTect2 algorithm (https://software.broadinstitute.org/gatk/documentation/tooldocs/3.8-0/org_broadinstitute_gatk_tools_walkers_cancer_m2_MuTect2.php).

After mutation calling, several filter criteria were used and excluded: (a) Germline mutations with ≥30% AFs in both PBL DNA and ctDNA, (b) Synonymous variants, (c) Variants with low depth (<500× in ctDNA). (d) Variants with less than 5 high-quality sequencing reads (mapqthres >30, baseqthres >30) in ctDNA (e) >1% samples found in single nucleotide polymorphism (SNP) databases (dbsnp, https://www.ncbi.nlm.nih.gov/projects/SNP/; 1000G, https://www.1000genomes.org/; ESP6500, https://evs.gs.washington.edu/; ExAC, http://exac.broadinstitute.org/). These remaining variants were identified as high-confidence somatic mutations. Clonal hematopoietic mutations, including those in *DNMT3A*, *IDH1* and *IDH2*, as well as specific alterations within *ATM*, *GNAS* and *JAK2* were also filtered previously described.[18]

2.5 | Statistical analysis

Continuous variables are expressed as the mean (±standard deviation [SD]) and were compared using Mann-Whitney U test. Categorical variables were compared using the χ^2 or Fisher's exact test. Univariate logistic regression analysis was used to investigate the risk factors of MVI. All variables significantly predicting MVI were subjected to multivariate logistic regression analysis. Receiver operating characteristic (ROC) curve was used to identify the optimal ctDNA cutoff value for the determination of MVI status. The association between variables and RFS were calculated using the Kaplan-Meier method and compared using the log-rank test. Multivariate Cox proportional hazards regression analysis was performed to evaluate the independent prognostic factors of RFS. In all analyses, $P < .05$ indicated statistical significance. All analyses were performed using IBM SPSS (version 23.0) and GraphPad Prism (version 6.01) software.

3 | RESULTS

3.1 | Clinicopathological characteristics of patients

The clinicopathological characteristics of the training and validation cohorts are listed in Table 1. In the training cohort, the average age at diagnose was 51.6 years old, and this cohort only encompassed three female patients (6.1%). No infection sign of HCV was found for patients in the training cohort, while 45 (91.8%) demonstrated positive HBV surface antigen (HBs-Ag) in blood test, of which 21 (42.9%) were detected with $>10^4$ IU/mL HBV-DNA. Via pathological examination, 29 patients (59.2%) were deemed MVI positive. The clinicopathological characteristics of patients in the validation cohort showed no significant difference in comparison with the training cohort.

3.2 | CtDNA detection and genomic landscape of HCC patients

In the training cohort, 260 somatic mutations were identified in ctDNA from 40 patients (81.6%) with the median of 5 (ranged from 1 to 34). The most recurrent mutant genes included *TP53* (55.1%), *TERT* (16.3%), *AXIN1* (12.2%), *LRP1B* (10.2%) and *CTNNB1* (10.2%) (Figure 1A). For *TP53* with extremely high mutant frequency, we further depicted the distribution of specific mutational sites and highlighted the recurrence of R249 mutation (Figure S1). According to previous reports, this mutation is closely associated with aflatoxin exposure and HBV infection,[19,20] and all of patients with *TP53* R249S mutations were identified with positive HBs-Ag in this cohort. The allele frequency (AF) range with the most mutations was 1%-10%, followed by 0.1%-1%, while only 33 (12.7%) and 31 (11.9%) mutations were located in 0.01%-0.1% and > 10% respectively (Figure 1B).

Parallel sequencing of tumour tissue samples was performed for patients in the training cohort, and somatic mutations were identified in all tumour tissues (n = 49). Similar to plasma results, *TP53* (65.3%), *TERT* (34.7%), *AXIN1* (20.4%), *CTNNB1* (18.4%) and *LRP1B* (10.2%) were frequently mutated, and the median number of somatic mutations was 6 per sample (ranged from 1 to 39) (Figure S2). The distribution of mutational sites in *TP53* was also quite concordant with plasma (Figure S1). The mutational landscape of tumour tissue was further compared with MSK-IMPACT Clinical Sequencing Cohort (MSKCC) which encompassed 85 HCC cases using panel deep sequencing.[21] As the results, the population frequencies of *TP53* (65.3% vs 27.1%) and *AXIN1* (20.4% vs 7.1%) in the training cohort were significantly higher than that in MSKCC, while an inferior frequency of *CTNNB1* (18.4% vs 37.6%) was seen in our cohort, possibly implying the disparate oncogenesis between Chinese and Caucasian patients based on genomic level (Figure S3). For 40 patients with somatic mutations in ctDNA, at least one ctDNA mutation was also detected in paired tissue sample in 39 patients (97.5%) (Figure 2). We defined the concordance rate for each patient as: the ctDNA mutations verified in paired tissue/the total ctDNA mutations, the perfect concordance was identified in 27 patients (67.5%), while the considerable mismatch of ctDNA mutations in paired tissues, such as

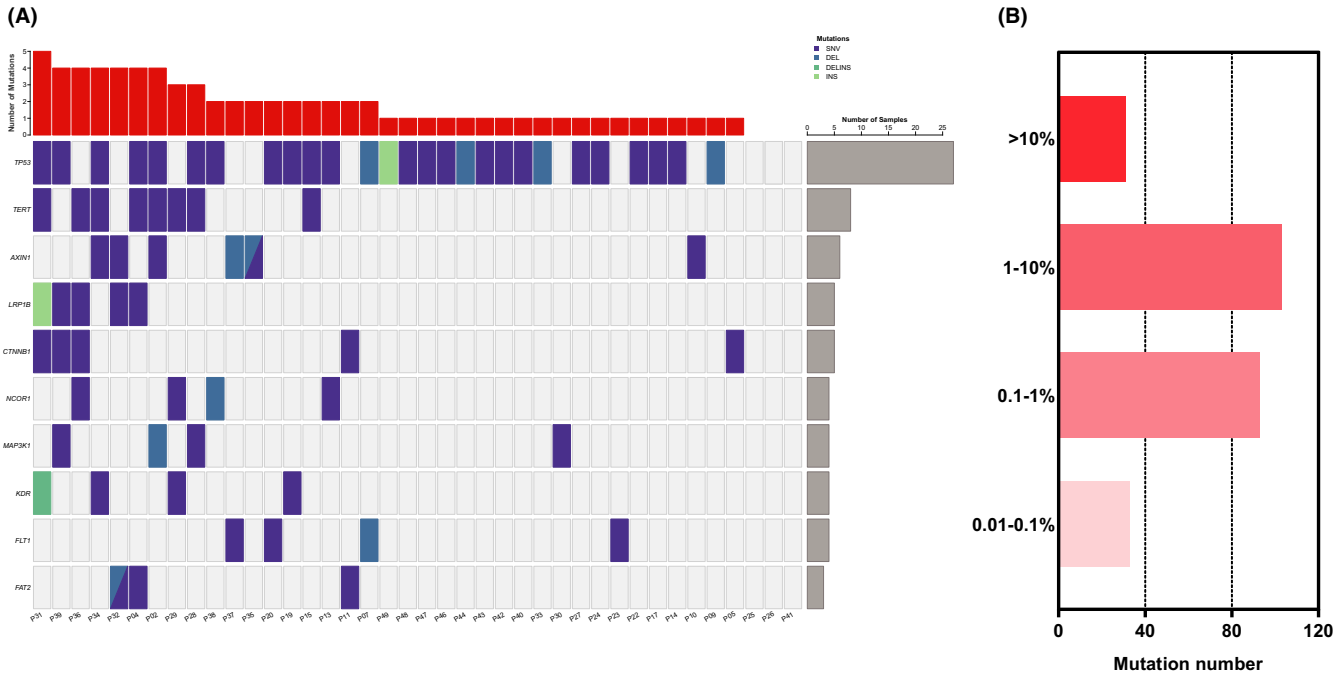

FIGURE 1 Genomic landscape of ctDNA in the training cohort. (A) Heatmap illustrating the common mutant genes of ctDNA. Only genes that mutated in over three patients were included. Each column represents a single patient. Different colours indicate diverse mutation types. The right bars represent the frequencies of each gene. The upper bars sum the total number of mutations only exhibited in the heatmap (not the overall mutation number) for each patient. (B) The distribution of AF for the overall ctDNA mutations. AF, allele frequency

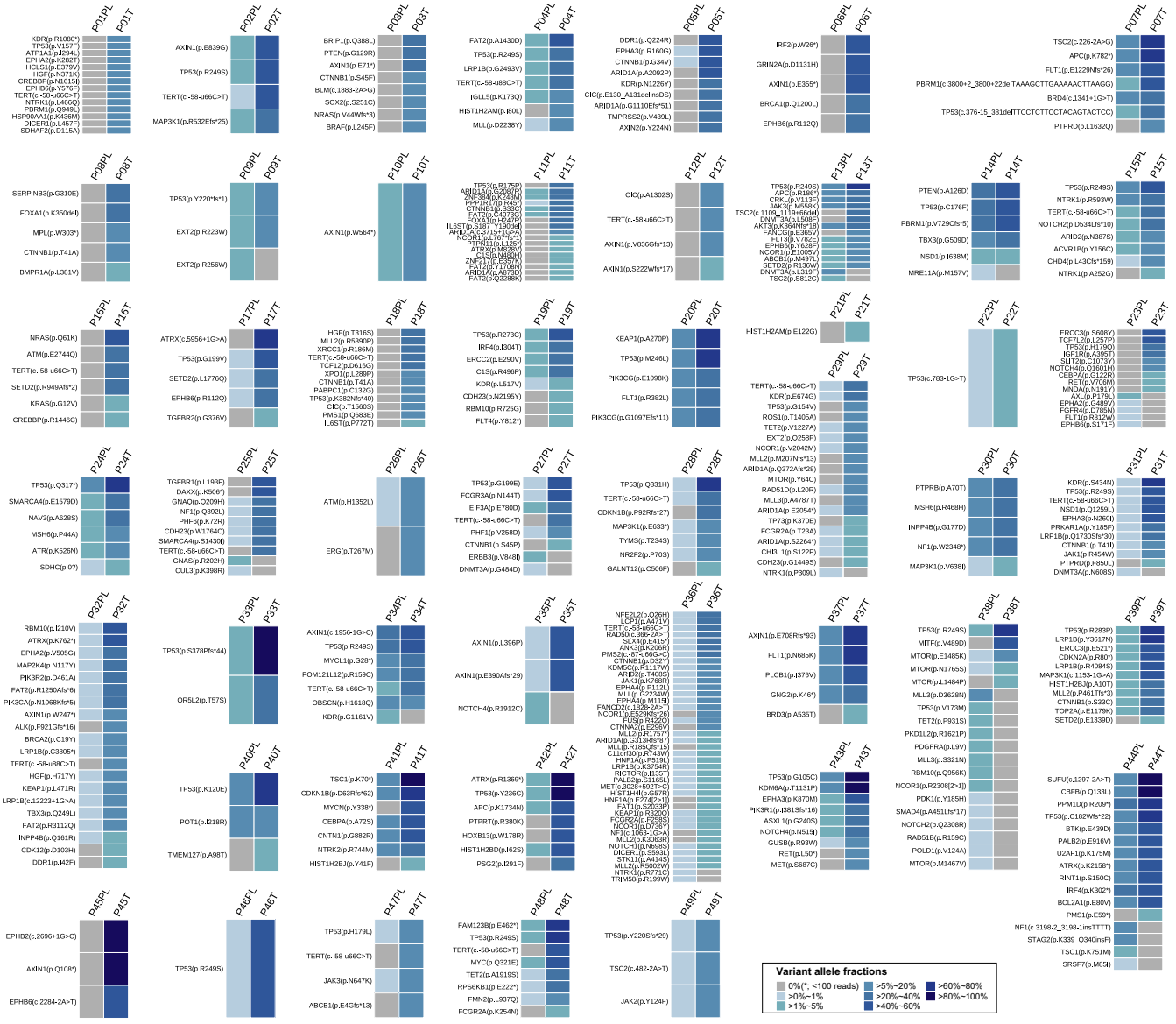

FIGURE 2 Somatic mutations found in paired ctDNA and resected tumour tissue in the training cohort. Heatmaps indicate the AF of the somatic mutations (blue, see colour key) or their absence (grey) in the 49 pairs of tumour tissue and ctDNA. T, tissue; PL, plasma

P23 and P38, was quite likely attributed to the intratumoral spatial heterogeneity (Figure 2; Figure S4A). Totally 85.8% of ctDNA mutations (223/260) were validated in paired tumour tissues, indicating ctDNA-based liquid biopsy a sensitive method to trace tumour-derived genomic alterations (Figure S4B).

3.3 | Correlation between MVI status and genomic features

Followed exploration about the genomic discrepancy between MVI positive and negative subgroups in the training cohort identified that all of ctDNA negative patients were absent with MVI, and the ctDNA detectability reached statistical significance between MVI positive and negative subgroups (100% vs 55%, adjusted χ^2 = 13.1, P < .001) (Figure 3). However, the blood tumour mutation burden (TMB) suggested no significant difference between two groups, neither did tissue TMB (Figure S5A). The maximal variant AF of ctDNA exhibited superior level in MVI positive patients (P < .001, Figure S5B).

Furthermore, we analysed the specific mutational spectrum in the MVI positive and negative groups in the training cohort. Only genes with >5% population frequencies were included. In plasma, several tumour suppressor genes, such as *APC*, *TSC2* and *MAP3K1*, were exclusively mutated in MVI positive patients, but did not reach statistical significance because of the moderate sample size (Figure 4A). The frequency of *TP53* was significantly higher in the MVI-positive group than in the MVI-negative group (79.3% vs 36.4%, P = .03, Figure 4A). Similarly in tissue samples, *TP53* was the only gene suggesting significant discrepancy between MVI positive and negative groups (82.8% vs 40.0%, P = .002, Figure 4B).

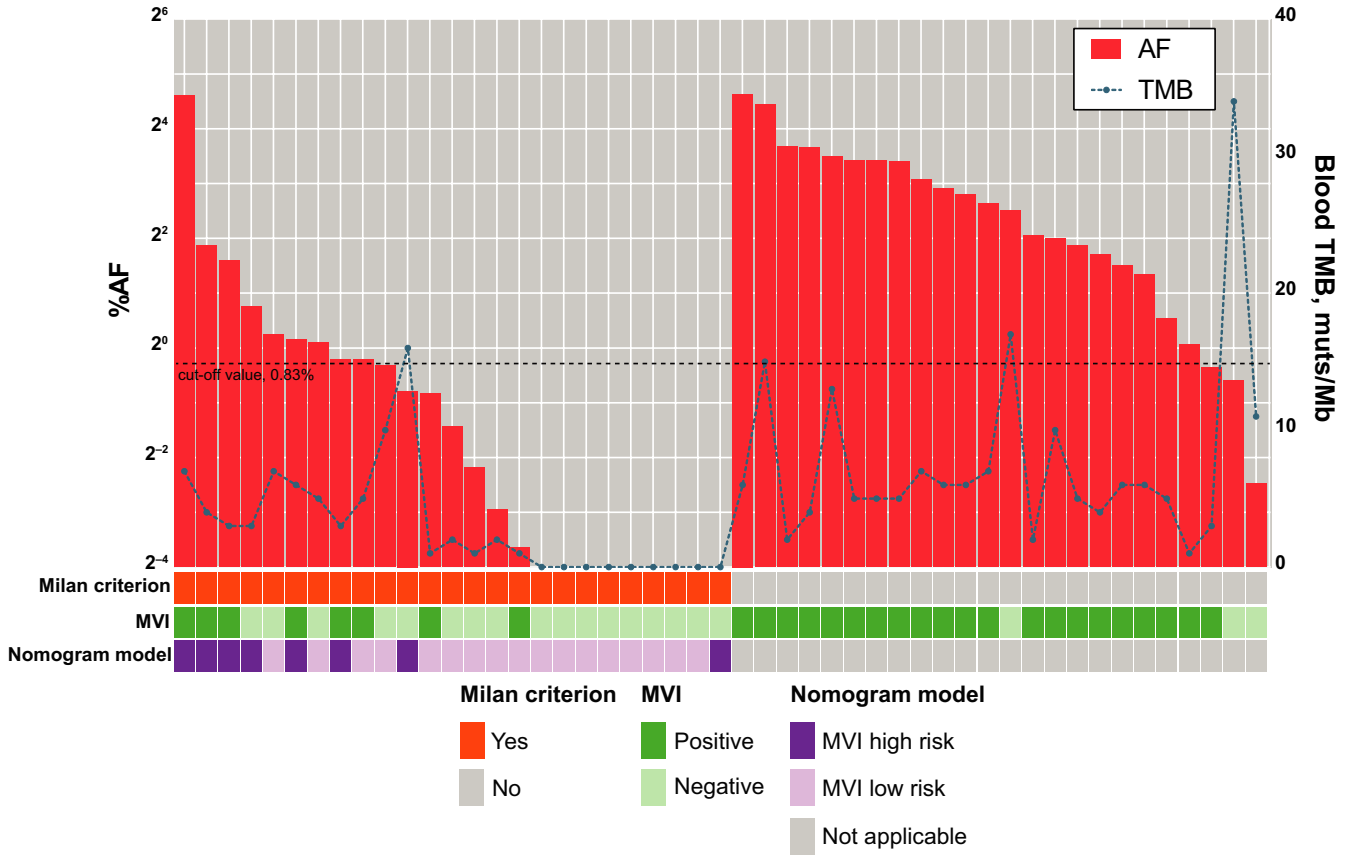

FIGURE 3 Correlation between ctDNA parameters, nomogram model and MVI status in the training cohort. The upper column chart and broken line graph indicates the distribution of the maximal AF and blood TMB respectively. The horizontal black broken line indicates the cuf-off value of ctDNA AF in distinguishing MVI high and low risk according to ROC analysis. The nomogram model encompass AFP, HBV-DNA, platelet count and imaging parameters to evaluate the MVI risk for HCC patients who met the Milan criteria. Patients who are inconsistent with the Milan criteria are labelled as 'Not applicable'. AF, allele frequency; TMB, tumour mutation burden

3.4 | Preoperative prediction of MVI via ctDNA parameters

Having shown that ctDNA AF was quite differentiated based on MVI status, we further evaluated its predictive performance with multiple clinicopathological characteristics excluding those with extremely unbalanced distribution (gender, HBs-Ag, and Child-Pugh grade). In univariate logistic regression analysis, only ctDNA AF (HR = 2.42, 95% CI 1.28-4.56, P = .01) and tumour size represented by the largest tumour diameter (HR 1.74, 95% CI 1.22-2.47, P < .001) were associated with the presence of MVI. Multivariate analysis encompassing both variables identified ctDNA AF (HR 2.15, 95% CI 1.09-4.24, P = .03) as the only independent risk factor for MVI status (Table 2).

Lei et al had developed a nomogram model which was an integrated score system using AFP, HBV-DNA, platelet count, and imaging parameters to predict MVI for HCC patients who met the Milan criteria.[12] To assess the performance of ctDNA AF against the nomogram model in preoperative prediction of MVI, the optimal cuf-off value for identifying the risk of MVI was firstly identified via ROC analysis in the training cohort. The area under the curve (AUC) was 0.92 (95% CI, 0.84-0.99, P < .001), indicating again the favourable efficacy of ctDNA in predicting MVI status (Figure S6). The cut-off value of ctDNA AF was set as 0.83% with the sensitivity of 89.7% and specificity of 80.0%: patients with ctDNA AF >0.83% were defined as high-risk MVI, while the others were deemed low-risk MVI. Particularly for 25 patients who met the Milan criteria in this cohort, the positive predictive value (PPV) and negative predictive value (NPV) of ctDNA AF was 66.7% and 87.5%, respectively, and the overall accuracy was 80.0%. As comparison, the PPV and NPV of nomogram model was 67.5% and 82.4%, respectively, and the overall accuracy was 76.0% (Figure 3). For HCC patients who met the Milan criteria, the performance of ctDNA was not inferior to the nomogram model. Notably, for patients who did not meet the Milan criteria, ctDNA presented a prediction accuracy of 91.7% (PPV: 95.2%; NPV: 66.7%) (Figure 3). Further evaluation was performed in the validation cohort, and the overall accuracy was 83.3% (sensitivity: 78.6%; specificity: 81.8%; PPV: 84.6%; NPV: 75.0%) (Table S2). Altogether, these data suggested that ctDNA was a potential biomarker to predict MVI preoperatively and might serve as a universal method in clinical practice in spite of the Milan criteria.

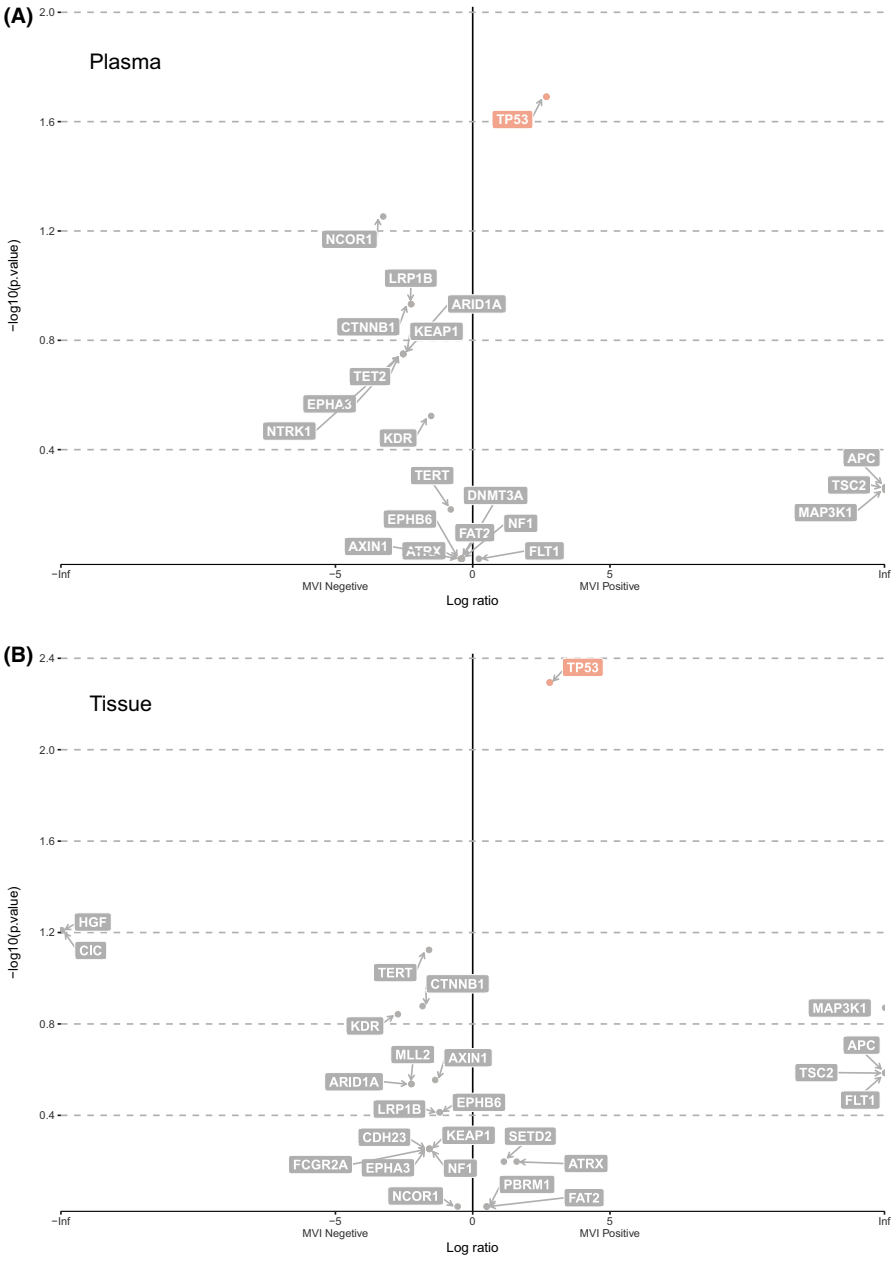

FIGURE 4 Significantly enriched mutant genes of ctDNA (A) and tumour tissue (B) in MVI positive and negative patients in the training cohort. Significantly enriched mutant genes is determined by χ^2 or Fisher's exact test. Only genes that mutated in over three patients were included in the analysis. Genes on the right and left of y-axis demonstrate superior population frequencies in MVI positive and negative patients respectively. Gene with $P < .05$ is identified with statistical significance and labelled as red box

3.5 | Effects of clinicopathological characteristics and ctDNA detection on the prognosis of operable HCC

As MVI status was deemed an effective prognostic factor for operable HCC and the tight association between ctDNA and MVI was revealed in this study, we hypothesized that preoperative ctDNA was also of prognostic value. To verify our hypothesis, all recorded clinicopathological characteristics (also excluding those with extremely unbalanced distribution) and ctDNA AF (cut-off value for high and low level: 0.83%) were tested in uniform process. Via Kaplan-Meier analysis, MVI status (HR = 4.91, 95% CI 1.64-14.70, $P = .005$), ctDNA AF (HR = 3.12, 95% CI 1.04-9.42, $P = .043$), AFP level (HR = 7.89, 95% CI 2.45-25.45, $P = .001$), Edmondson-Steiner classification (HR = 3.90, 95% CI 1.06-14.43, $P = .041$) and BCLC staging (HR = 30.56, 95% CI 6.23-150.0, $P < .001$) demonstrated significant association with RFS in the training cohort (Figure 5A,B; Figure S7). While only ctDNA AF (HR = 8.40, 95% CI 1.18-59.91, $P = .034$) reached statistical significance in the validation cohort, the prognostic performance of these factors were repeatable in the total cohort (Figure 5A,B; Figure S7). As we had shown that ctDNA was associated with the MVI status of the resected specimen, further multivariate analysis was conducted with these factors available preoperatively (ctDNA AF, AFP level and BCLC staging) in the total cohort. We found that all three features (ctDNA AF, HR = 4.56, 95% CI 1.02-20.50, $P = .048$; AFP level, HR = 4.65, 95% CI 1.45-14.89, $P = .010$; BCLC staging, HR = 2.78, 95% CI 1.03-7.51, $P = .045$) were independent risk factors for MVI (Figure 5C). Altogether, these results suggest that preoperative ctDNA determination could not only predict for the presence of MVI in post-operative pathological

TABLE 2 Univariate and multivariate analysis for MVI prediction with multiple clinicopathological variables and ctDNA parameters

	Univariate analysis		Multivariate analysis	
Variable	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**
Age, years (>50 vs ≤50)	1.64 (0.52-5.19)	.40	—	—
HBV-DNA, IU/mL (>10^4 vs ≤10^4)	0.82 (0.26-2.61)	.74	—	—
MELD score	0.44 (0.12-1.65)	.22	—	—
ICGR15, %	0.95 (0.85-1.05)	.30	—	—
Albumin, g/L	0.98 (0.87-1.10)	.69	—	—
PLT,10^9/L	1.01 (1.00-1.02)	.14	—	—
Total bilirubin, μmol/L	1.01 (0.90-1.13)	.89	—	—
Direct bilirubin, μmol/L	1.04 (0.81-1.32)	.77	—	—
PT-INR	0.12 (0-34.56)	.46	—	—
ALT, U/L	1.01 (0.99-1.03)	.54	—	—
AST, U/L	1.01 (0.99-1.04)	.38	—	—
AFP, ng/mL	1.00 (1.00-1.00)	.22	—	—
CA19-9, IU/mL	0.99 (0.96-1.02)	.40	—	—
ctDNA AF, %	2.42 (1.28-4.56)	.01*	2.15 (1.09-4.24)	.03*
Blood TMB, muts/Mb	1.00 (0.91-1.10)	.94	—	—
Tumour size, cm	1.74 (1.22-2.47)	.00*	1.17 (0.80-1.70)	.43
Tumour number (multiple vs, single)	0.96 (0.23-3.95)	.95	—	—
Tumour capsule (incomplete vs complete)	1.88 (0.33-10.79)	.48	—	—
Cirrhosis (positive vs negative)	2.11 (0.55-8.01)	.28	—	—
BCLC staging (0/A vs B/C)	1.89 (0.41-8.03)	.44	—	—

Abbreviation: MELD, model for end-stage liver disease; ICGR15, indocyanine green retention rate at 15 min; PLT, platelet count; PT-INR, prothrombin time-international normalized ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, alpha fetoprotein; CA19-9, carbohydrate antigen 19-9; AF, allele frequency; TMB, tumour mutation burden.

*Statistical significance.

examination, but also serve as a potential prognostic factor in addition to other routine clinical tests.

4 | DISCUSSION

Herein we report a study about stating the genomic landscape of HCC via ctDNA-based liquid biopsy and evaluating the performance of preoperative ctDNA detection in predicting MVI status of HCC. Results showed that about 86% of ctDNA mutation could be validated in paired tumour tissue samples, and the elevated ctDNA AF could independently predict the presence of MVI identified by postoperative pathological examination. Besides, ctDNA AF was valuable in estimating recurrence risk, and the prognostic performance was still significant in multivariate analysis.

Currently, the diagnosis of MVI mainly depends on microscopic microvascular analysis of HCC specimens following surgery. Although MVI can accurately predict the prognosis of HCC after surgery, it cannot currently be used preoperatively to guide the choice of treatment. The preoperative evaluation of MVI could dramatically impact the decision-making during surgical operation, such as the selection of anatomical or non-anatomical resection,[22,23] and determination of the most suitable patients for liver transplantation.[24] Currently, many biomedical efforts, including imageology, genetic characteristics, circulating tumour markers, blood routine and biochemistry, and other clinical characteristics, have been explored in the preoperative evaluation of MVI,[9,12,13] but most are too complicated to be practically applied and the performance is still far from satisfaction. It is imperative to explore a more simplified method with satisfactory efficacy to predict the presence of MVI. Some proof-of-principle studies have demonstrated that ctDNA has significant advantages in molecular profiling and monitoring for cancer patients,[25-27] but the application of ctDNA in preoperative management is limited. Considering the biological nature of ctDNA,

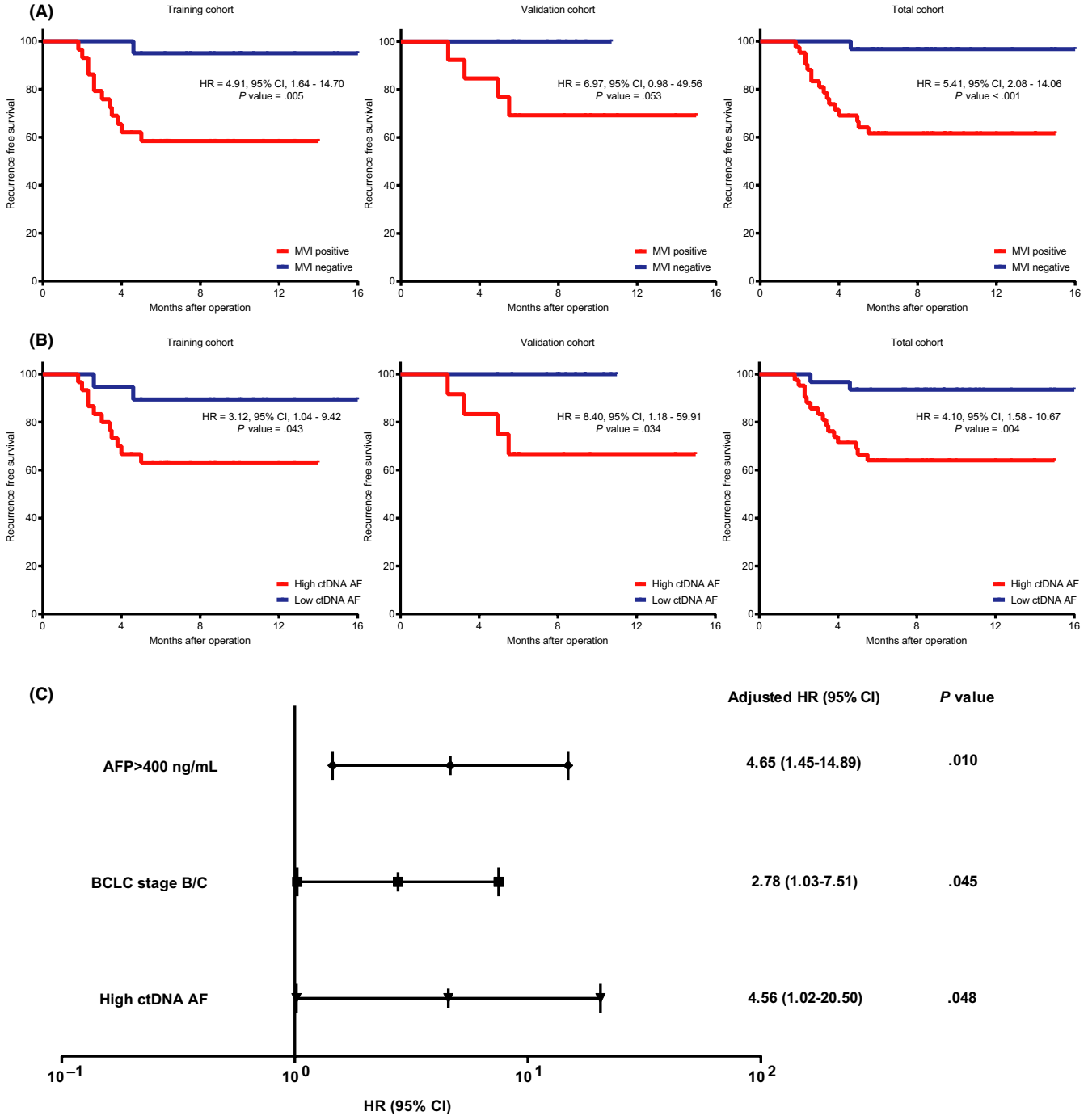

FIGURE 5 Survival analysis for patients with different clinicopathological characteristics and ctDNA AF. The patients are grouped by MVI status (A) and maximal ctDNA AF (B). The results of both training and validation cohort, as well as the total cohort, are exhibited. (C) Multivariate COX regression analysis reveals the independent risk factors for postoperative recurrence. Only variables suggesting statistical significance in univariate Kaplan-Meier analysis are included in the COX model, and the analysis is based on the data from the total cohort. HR, hazard ratios

we inferred the possible correlation between MVI and ctDNA level which is evidentially verified in this study. Our findings not only expands the potential applicability of ctDNA detection in preoperative decision-making, but also enable the improvement of subsequent medical treatment given that ctDNA can profile the genomic landscape of cancer patients. Moreover the finding that the MVI predictive performance of ctDNA is not subject to the Milan criteria highlights the universal applicability of ctDNA and thus may benefit a broader population than traditional models.

As one of the most malignant cancer types, the five-year relative survival rates of localized, regional and metastatic HCC are approximate 30%, 10% and 3% respectively.[28] Although the overall survival is quite poor, the localized/regional HCC still suggest a more favourable prognosis than metastatic HCC, which reminds us the necessity

to develop and improve early screening and diagnostic methods, especially for those who have a high risk of developing HCC, such as cirrhosis patients.[29,30] Currently, the screening and diagnostic criteria are mainly based on hepatitis virus detection, ultrasonography and AFP test. Although these modalities have suggested prominent advancement in overall survival because of early detection and receipt of curative therapies,[31] still a lot of improvements are expected. In this study, the result that the sensitivity of ctDNA in early stage HCC is over 80% implies ctDNA may be a feasible approach in the early screening and detection of HCC, which is in cooperation with recent reports that ctDNA methylation[15,32] and the combination of ctDNA mutation and other serum markers[33] are tested with decent performance.

Tissue biopsies are usually non-essential for the diagnosis of HCC. For patients who are not eligible for tumour resection, tissue specimens are commonly unavailable for molecular profiling, which consequently hinder the precise management. Ng et al have demonstrated that the use of cfDNA for genetic profiling when biopsy is unavailable may be feasible in HCC patients with high disease burden.[34] Similarly, even in operable patients with relatively low disease burden, the genomic concordance between ctDNA and tumour tissue is still satisfactory according to our study. Based on the results, ctDNA detection can be utilized as prior knowledge for the resected tissue to improve the accuracy of genomic profiling. But notably, still a fraction of plasma or tissue mutations were private to specific specimens in cross validation, which may be attributed to tumour heterogeneity and subclonal evolution: one hand, single-site tissue biopsy cannot reflect the whole genomic landscape of tumour for that some subclonal mutations which are detectable in plasma ctDNA only exist in a moderate fraction of tumour cell; on the other hand, the release of ctDNA into circulation from subclonal cell population are generally more deficient than clonal cell population, leading to the fact that the information content of some subclonal mutations in the plasma are below the detection limit. To address the problems and further validate the capacity of ctDNA in revealing the genomic landscape of HCC, multi-regional sampling and improvement of sequencing platforms are necessarily needed.

The primary limitation of our study is the moderate cohort size, which leads to the inadequate evaluation of MVI risk factors and prognostic factors. Besides, the detection sensitivity for ctDNA mutations need to be further enhanced for that still a fraction of tumour-derived mutation, mainly subclonal mutations with inferior AF in the tissue samples, are absent in ctDNA profiling. Despite these limitation, we provide strong evidences that somatic mutations can be reliably detected in HCC patients with relatively low disease burden, and demonstrate ctDNA as a novel, highly sensitive and reliable biomarker to predict MVI preoperatively. In practice, we hope ctDNA can serve to guide the choice for the most appropriate surgery or treatment for HCC patients, and enable provision of more precise treatment strategies and ultimately better clinical outcomes. Our results also support further investigations into the clinical utility of ctDNA with a larger cohort of patients.

4.1 | Conclusion

CtDNA can serve as an independent risk factor of MVI for operable HCC and help determining precise treatment strategies. The integration of ctDNA in the management of operable HCC may achieve better clinical outcomes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

LeDu Zhou takes responsibility for the integrity of the work as a whole, from inception to published article. Dong Wang performed the research. Xi Hu and Guo Long collected the blood and tissue samples. Ke Ye and Liang Xiao collected the patients clinical data. Yaping Xu, Lifeng Li, Lianpeng Chang, Sha Wang, Yanfang Guan and Qiongzhi He analysed the data. Yaping Xu, Xuefeng Xia and Ledu Zhou designed the research study and wrote the paper. Jennifer B. Goldstein, Xin Yi, Jianjun Zhang and Zhiming Wang contributed to the design of the study and edited the manuscript. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Researchers who provide a methodologically sound proposal and attempt to achieve aims in the approved proposal can obtain individual participant data that underlie the results reported in this article from the corresponding author (zhould@csu.edu.cn) after publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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