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Research paper

Effect of surgical margin on recurrence based on preoperative circulating tumor cell status in hepatocellular carcinoma

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ABSTRACT

Background: High rates of recurrence after resection severely worsen hepatocellular carcinoma (HCC) prognosis. This study aims to explore whether circulating tumor cell (CTC) is helpful in determine the appropriate liver resection margins for HCC patients.
Methods: HCC patients who underwent liver resection were enrolled into training (n=117) or validation (n=192) cohorts, then classified as CTC-positive (CTC≥1) or CTC-negative (CTC=0). A standardized pathologic sampling method was used in the training cohort to quantify microvascular invasion (mVI) and the farthest mVI from the tumor (FMT).
Findings: CTC number positively correlated with mVI counts (r=0.655, P<0.001) and FMT (r=0.495, P<0.001). The CTC-positive group had higher mVI counts (P=0.032) and greater FMT P=0.008) than the CTC-negative group. In the CTC-positive group, surgical margins of >1 cm independently protected against early recurrence (training cohort, P=0.004; validation cohort, P=0.001) with lower early recurrence rates (training cohort, 20.0% *vs.* 65.1%, P=0.005; validation cohort, 36.4% *vs.* 65.1%, P=0.003) compared to surgical margins of ≤1 cm. No differences in postoperative liver function were observed between patients with margins >1 cm *vs.* ≤1 cm. Surgical margin size minimally impacted early postoperative HCC recurrence in CTC-negative patients when using 0.5 cm or 1 cm as the threshold.
Interpretations: Preoperative CTC status predicts mVI severity in HCC patients and is a potential factor for determining optimal surgical margin size to ensure disease eradication and conserve liver function. A surgical margin of >1 cm should be achieved for patients with positive CTC.
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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and leading causes of malignancy-related deaths worldwide [1,2]. Surgical resection remains one of the most effective treatments with curative potential [3]. Even after such surgery, the 5-year cumulative recurrence rate is 50–70%, which severely reduces the long-term survival of HCC patients. Early recurrence accounts for most (approximately 70%) recurrent cases [4], mainly due to postoperative minimal residual disease (MRD) [5,6]. Although a wider surgical margin may prevent early recurrence in HCC patients [7], too wide of a margin may leave insufficient liver parenchyma and lead to liver failure after resection [8]. Balancing operative safety and efficacy is necessary for surgically treating HCC patients. The optimal resection margin for surgically treating HCC is currently arbitrary and controversial [7, 9–11].

Many previous studies have addressed the prognostic significance of microvascular invasion (mVI), one of the most important related risk factors for early postoperative recurrence of HCC [12,13]. However, it is very difficult to assess the probability of mVI before

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Research in context

Evidence before this study

Hepatocellular carcinoma (HCC), which comprises the majority of liver cancer was a leading cause of death worldwide. The high rate of recurrence severely hampers the long-term survival of HCC patients after liver resection. Early recurrence, which is mainly caused by intrahepatic dissemination, accounts for nearly 70% of all recurrent cases. Circulating tumor cells (CTC), as a component of “liquid biopsy”, has shown potential clinical implications. Patients with positive CTC have higher risk of recurrence, shorter overall survival, and associate with poor clinical characteristics. CTC is a promising prognostic biomarker; however, it remains unclear whether CTC helpful in guiding the surgical plan.

Added value of this study

We found that CTC was associated with the severity of microvascular invasion (mVI) in the peritumoral tissue. For CTC-positive patients, 79.9% of mVI distributed within 1 cm from the tumor border, while 83.7% mVI distributed within 0.5 cm in CTC-negative patients. Preoperative CTC-positive patients showed significantly better prognosis with a surgical margin of $>$1 cm.

Implications of all the available evidence

In addition to the well-accepted role in metastasis, CTC could also help evaluate the severity of local dissemination in HCC patients. Preoperative CTC detection was a promising parameter to precisely determine the optimal surgical margin before liver resection, ensuring the safety and efficacy of operation.

operation so as to determine more precise surgical excision ranges preoperatively [14–16]. Circulating tumor cells (CTC) have garnered significant interest as the potential "seeds" that initiate tumor recurrence and metastasis [17–22], and our previous study demonstrated that preoperative CTC $\geq$2 predicts early postoperative HCC recurrence [23]. Moreover, the correlation between preoperative CTC status and vascular invasion was confirmed, implicating preoperative CTC status as a potential factor for evaluating the presence of mVI in HCC. However, no comprehensive study on the relationships among preoperative CTC status, mVI, and optimal resection margins for HCC patients has been performed.

In the current study, extensive research of mVI distribution in peritumoral tissues of HCC patients was performed using the multipoint sampling method, and the correlation between the number and distance of mVI dissemination and preoperative CTC status was explored. The impact of resection margin width on early recurrence and overall survival (OS) in HCC patients with different CTC statuses was further evaluated. Finally, optimal surgical margins for HCC patients with or without preoperative CTC were explored and validated in another independent cohort.

2. Materials and methods

2.1. Patients and follow-up

All patients were enrolled at the Zhongshan Hospital Fudan University (Shanghai, China). Inclusion criteria were as follows: available preoperative CTC detection data; no history of previous anticancer therapy; no history of other malignancies; lesions (single tumor or adjacent multiple tumors) that could be removed by one excised specimen; curative resection was defined as complete resection of all tumor nodules and the cut surface being free of cancer by histologic examination, without extrahepatic metastasis and cancerous thrombus in the portal vein (main trunk or two major branches), hepatic veins, or bile duct [24].

Exclusion criteria were tumor type other than HCC and withdrawal of informed consent.

Four hundred forty-eight patients underwent CTC testing before treatment for liver cancer during the study period. Of these, 139 patients were excluded based on [1] secondary liver cancer diagnosis (n=15) [2]; receiving antitumor therapy before registration (n=35) [3]; receiving HCC treatment other than resection during hospitalization such as transhepatic arterial chemotherapy and embolization (TACE), portal vein embolization (PVE), radiofrequency ablation (RFA), portal vein thrombectomy and systemic anti-tumor therapy (n=38) [4]; exhibiting extrahepatic metastasis (n=4) [5]; benign lesions (n=7) or other types of malignant tumors (such as intrahepatic cholangiocarcinoma, hepatic sarcomatoid carcinoma and hepatic neuroendocrine tumor) (n=40) according to histologic examination. The remaining 309 patients were finally enrolled and divided into two independent cohorts. First, 117 patients registered from February 2014 to October 2015 were grouped into the training cohort and a multiregional sampling method was used. Complete mVI data were used to explore mVI distribution and its correlation with preoperative CTC status. A second independent cohort including 192 patients hospitalized during August 2010 to January 2014 were retrospectively enrolled as the validation cohort. A study design schematic is shown in Fig. 1. The clinicopathologic characteristics of the patients in training and validation cohorts were summarized in **Table S1**. Approval for the use of human subjects was obtained from the research ethics committee of Zhongshan Hospital (No. 2011-62), and informed consent was obtained from each patient.

Adjuvant TACE would be recommend for patients who were assessed as at intermediate to high risk of recurrence according to our previous report (such as vascular invasion, or multiple tumors, or tumor size $>$5 cm, et al) [25]. No patient received adjuvant systemic therapy without the evidence of tumor burden. Patients were observed every 2–3 months in the first 2 years after surgery and then every 3–6 months thereafter. Serum α-fetoprotein (AFP) assay and abdominal ultrasonography were performed at each follow-up visit. Enhanced magnetic resonance imaging (MRI) or computed tomography (CT) of the abdomen was performed every 6 months. If intrahepatic recurrence and/or distal metastasis were clinically suspected based on symptoms or unexplained elevation of tumor marker levels, MRI, CT, or bone scans were performed immediately. Time to recurrence (TTR) was defined as the interval between treatment and intrahepatic recurrence or extrahepatic metastasis [26], and time to early recurrence (TTER) was defined as a TTR within 2 years [27]. The OS was defined as the interval between treatment and death by any cause or the last observation date. The follow-up period ended in December 2019.

2.2. CTC detection

Blood was preoperatively drawn from the peripheral vein of each patient. CTC analysis was performed using the CellSearch® system (Veridex, Raritan, NJ) as described in our former work [23] by clinical laboratory technicians blinded to patient clinical characteristics. CTC enumeration was expressed as the number of cells per 7.5 mL of blood, and positive CTC was defined as CTC $\geq$1.

2.3. Evaluation of surgical margin

Before formalin fixation, resected specimens were serially cut into 1 cm thick slices. The extent of the unfixed specimen resection margins was measured radially on each slice. Surgical margin was

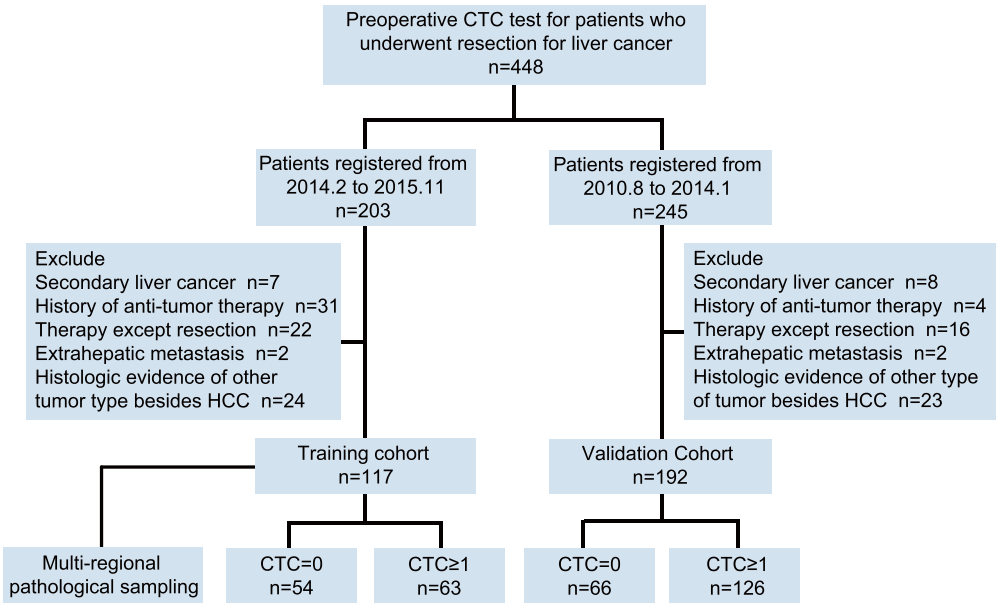

Fig. 1. The flow chart of the study design. (CTC, circulating tumor cell; HCC, hepatocellular carcinoma.).

defined as the shortest macroscopic distance from the edge of the tumor to the line of transection. In specimens with multiple tumors, the shortest resection margin of all tumors was considered the surgical margin.

2.4. Histologic evaluation of mVI

Seven points were sampled from resected specimens fixed in 10% formalin for pathological evaluation using a modified standard method [28,29] (Fig. 2A). Four perpendicular points containing both tumor and peritumor tissues were taken at the edge of tumor ($\leq$1 cm); three points were randomly sampled from nontumorous liver parenchyma covering distances of 1−2 m, 2−3 cm, and >3 cm away from the tumor edge (Fig. 2A). Each sample was 1.5 cm × 1.0 cm × 0.2 cm. Samples were then embedded in paraffin and serially sliced into sections. One section was randomly taken from each block for hematoxylin and eosin staining and evaluation of mVI, which was defined as tumor cells within a vascular space visible with microscopy [30], and the mVI counts were defined as the total numbers of vessels invaded by mVI in peritumoral area [31,32]. The farthest mVI from the tumor (FMT) was defined as the distance of the mVI detected in the farthest positive vessel from the tumor border, and the FMT in patients without mVI was recorded as "none". All sections were assessed by two independent pathologists blinded to patient characteristics, and disagreements were discussed at a microscope (Novel, Ningbo, China) until consensus was reached.

2.5. Postoperative liver function

To investigate liver restoration after resection, liver function tests were performed routinely preoperatively and on postoperative days (POD) 1, 3, 5, 7. Serum total bilirubin (TB), alanine transferase (ALT), aspartic acid transferase (AST), prothrombin time (PT) and international normalized ratio (INR) were recorded [33,34]. We adopted the definition of posthepatectomy liver failure (PHLF) by the International Study Group of Liver Surgery (ISGLS) [8,35], and Grade C PLF was defined as the patients with increased international normalized ratio (INR), concomitant hyperbilirubinemia on or after postoperative day 5 and requiring invasive treatment or vasoactive drugs.

2.6. Statistical analysis

Statistical analyses were performed with SPSS version 20.0 for Windows (IBM, Armonk, NY). Chi-square, Fisher's exact, Student's *t*, and Mann-Whitney *U* tests were used for comparing groups when appropriate. Pearson correlation test was used to analyze the correlation. ROC (receiver operator characteristic) curve analyses were used to evaluate the prediction value of CTC. TTER and OS curves were analyzed using the Kaplan-Meier method and compared using a log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazard regression model. Power calculation and sample size estimation were performed by PASS trial version. Differences with $P<0.05$ were considered statistically significant.

2.7. Role of funders

The funders who provided the funding had no role in study design, data collection, data analyses, interpretation or writing of report.

3. Results

3.1. The distribution of mVI in peritumoral tissues of HCC patients assessed by multiregional sampling

The status of mVI was evaluated in 117 HCC patients receiving multi-regional pathological sampling as described in the method (Fig. 2A). After examining all pathological sections, 58 of 117 patients (49.6%) were diagnosed as mVI-positive. Two hundred three micro-vessels were detected with tumor micro-emboli, and mVI-positive micro-vessel prevalence was 189/203 (93.1%) in the portal vein, 12/203 (5.9%) in the hepatic vein, and 2/203 (1.0%) in the hepatic artery (Fig. 2B). The distribution of all mVI in peritumoral tissues was shown in Fig. 2C, and the details of FMT in 58 patients with mVI presence was shown in Fig. 2D.

Preoperative CTC status positively correlated with the existence and severity of mVI in peritumoral tissues

In the CTC-positive group, 35 patients (55.6%, 35/63) were mVI-positive, with total mVI counts of 154. In the CTC-negative group, 23 patients (42.6%, 23/54) were mVI-positive, with total mVI counts of 49. Meanwhile, 63.0% (97/154) mVI was located within 0.5 cm of the

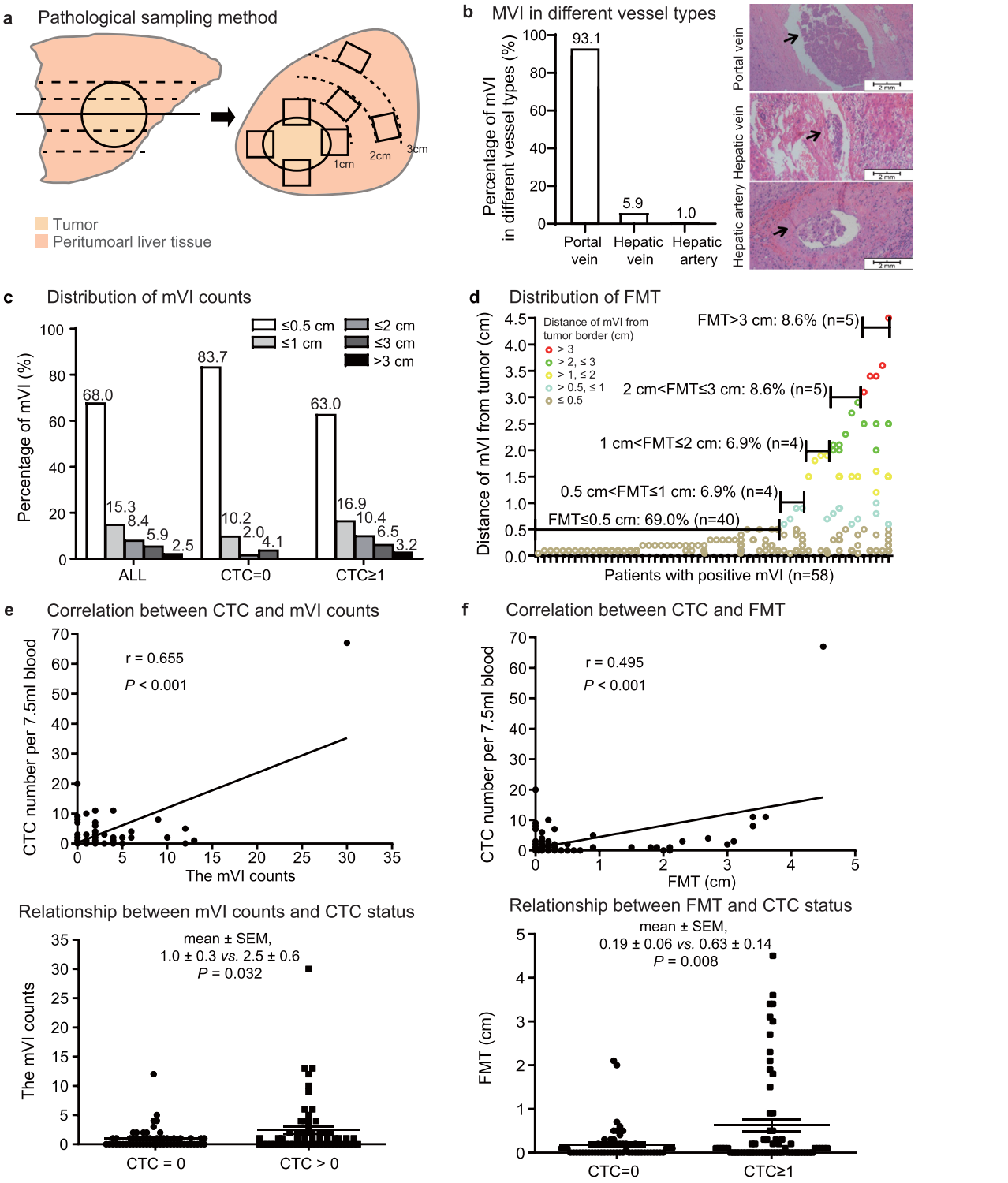

Fig. 2. Distribution of mVI in peritumoral tissues. (A) Multiple regional sampling method from the resected specimen. (B) The distribution of mVI in different vessel types and the typical image of mVI presented in portal vein, hepatic vein and hepatic artery. Specimen was peritumoral tissues. Scale bar represented 2 mm. Arrow represented the mVI in vessels. (C) Distribution of mVI counts among all mVI. (D) FMT distribution in mVI positive patients. (E) Linear correlation between mVI counts and CTC (upper); Relationship between mVI counts and CTC status (lower). (F) Linear correlation between FMT and CTC (upper); Relationship between FMT counts and CTC status (lower). (CTC, circulating tumor cell; FMT, the farthest mVI from the tumor; mVI, microvascular.) Pearson r correlation tests and Mann-Whitney *U* tests were used.

tumor edge in the CTC-positive group, which was significantly lower compared with 83.7% (41/49) in the CTC-negative group ($P<0.001$, chi-square test, Fig. 2C). Preoperative CTC level positively correlated with the number of mVI ($r=0.655$, $P<0.001$, Pearson correlation test, Fig. 2E), and the number of mVI in the CTC-positive group was significantly higher than that in the CTC-negative group (mean 2.5±0.6 *vs.* 1.0±0.3 counts per patient, $P=0.032$, Mann-Whitney *U* test, Fig. 2F). We also found that preoperative CTC level was positively correlated with FMT ($r=0.495$, $P<0.001$, Pearson correlation test, Fig. 2G), and the FMT of mVI in CTC-positive patients was farther than that in CTC-negative patients (mean 0.63±0.14 cm *vs.* 0.19±0.06 cm, $P=0.008$, Mann-Whitney *U* test, Fig. 2H).

The quantity (counts more than 5) and disseminated distance (FMT >1 cm) were used to evaluate the severity of mVI according to a previous report [28], and the performance of CTC detection in assessing mVI severity was further explored. We found that CTC was able to predict mVI counts >5 [AUC (area under ROC curve)=0.744, 95% confidence interval (CI) 0.655 to 0.820; sensitivity 88.9%, specificity 49.1%, *P*=0.003, Z-test, Figure S1A] and FMT >1 cm (AUC=0.733, 95% CI 0.644 to 0.811; sensitivity 85.7%, specificity 50.5%, *P*=0.001, Z-test, Figure S1B).

3.2. Effects of surgical margin on early recurrence of HCC based on preoperative CTC status

The mean FMT in CTC-positive patients was 0.63±0.14 cm, and 79.9% of mVI (123/154) was detected within 1 cm of the tumor border, while the FMT of CTC-negative patients was 0.19±0.06 cm with 83.7% of mVI (41/49) distributed within 0.5 cm of the tumor border. These data implied that a surgical margin >1 cm might be optimal for CTC-positive patients and a margin of 0.5 cm might be adequate for CTC-negative patients in order to prevent development of mVI-associated MRD as well as preserve maximum liver parenchyma. Thus, we divided HCC patients into three surgical margin subgroups [margin$^{\leq 0.5}$ (margin ≤0.5 cm), margin$^{0.5-1}$ (0.5 cm< margin ≤1 cm), and margin$^{>1}$ (margin >1 cm)] to further evaluate the effects of surgical margin on early recurrence based on preoperative CTC status. The detail and the comparison of the baseline characteristics were shown in Table 1, and no significant difference was found among three groups of different surgical margins.

Among 63 patients in the training cohort with detectable CTC, 34 (54.0%) developed early postoperative tumor recurrence (≤2 years). Univariate analysis and unadjusted hazard ratios (HRs) for TTER were calculated in preoperative CTC-positive patients. Large tumor size, multiple tumors, vascular invasion, and AFP >400 ηg/mL were correlated with early recurrence in HCC patients with detectable CTC (Table 2). Kaplan-Meier analysis (medians presented) showed that patients in the margin$^{>1}$ group had significantly longer TTER than those in the margin$^{\leq 0.5}$ (not reached *vs.* 5.9 months, *P*=0.004, log-rank test) and margin$^{0.5-1}$ (not reached *vs.* 18.7 months, *P*=0.025, log-rank test) groups, while no significant difference in early recurrence between margin$^{\leq 0.5}$ and margin$^{0.5-1}$ groups was seen (*P*=0.330,

Table 1
Comparison of demographics and clinicopathologic characteristics among patients with different margin in the training cohort

Characteristics	Surgical margin ≤ 0.5 cm (n = 51)	Surgical margin 0.5-1 cm (n = 32)	Surgical margin > 1 cm (n = 34)	*P* value
Sex				
Female, n (%)	9 (18)	3 (9)	4 (12)	0.525
Male, n (%)	42 (82)	29 (92)	30 (88)	
Age (mean ± SD), yr	55.8 ± 11.8	56.7 ± 11.7	50.2 ± 12.4	0.060
CTC Count (mean ± SD)	1.9 ± 2.8	4.2 ± 12.1	1.2 ± 2.4	0.300
Tumor diameter (mean ± SD), cm	6.2 ± 4.3	5.4 ± 3.7	4.7 ± 2.9	0.201
Tumor number				
Single tumor, n (%)	31 (61)	25 (78)	28 (82)	0.062
Multiple tumors, n (%)	20 (39)	7 (22)	6 (18)	
Tumor capsule				
Incomplete, n (%)	19 (37)	8 (25)	17 (50)	0.111
Complete, n (%)	32 (63)	24 (75)	17 (50)	
Edmondson grades				
I/II, n (%)	31 (61)	19 (59)	22 (65)	0.896
III/IV, n (%)	20 (39)	13 (41)	12 (35)	
Vascular invasion				
No invasion, n (%)	21 (41)	16 (50)	17 (50)	0.637
Vascular invasion, n (%)	30 (59)	16 (50)	17 (50)	
Cirrhosis				
No, n (%)	25 (49)	17 (53)	15 (44)	0.764
Yes, n (%)	26 (51)	15 (47)	19 (56)	
HBsAg				
Negative, n (%)	5 (10)	5 (16)	5 (15)	0.688
Positive, n (%)	46 (90)	27 (84)	29 (85)	
Total bilirubin				
≤ 20.4 μmol/L, n (%)	44 (86)	31 (97)	33 (97)	0.099
> 20.4 μmol/L, n (%)	7 (14)	1 (3)	1 (3)	
Albumin				
< 35 g/L, n (%)	1 (2)	2 (6)	2 (6)	0.552
≥ 35 g/L, n (%)	50 (98)	30 (94)	32 (94)	
ALT				
≤ 50 U/L, n (%)	36 (71)	26 (81)	22 (65)	0.318
> 50 U/L, n (%)	15 (29)	6 (19)	12 (35)	
γ-GT				
≤ 60 U/L, n (%)	29 (57)	21 (66)	21 (62)	0.720
> 60 U/L, n (%)	22 (43)	11 (34)	13 (38)	
AFP				
≤ 400 ng/mL, n (%)	34 (67)	25 (78)	25 (74)	0.510
> 400 ng/mL, n (%)	17 (33)	7 (22)	9 (26)	
Child-pugh classification				
A, n (%)	50 (98)	32 (100)	33 (97)	0.643
B, n (%)	1 (2)	0 (0)	1 (3)	
Adjuvant TACE				
No, n (%)	35 (69)	24 (75)	25 (74)	0.792
Yes, n (%)	16 (31)	8 (25)	9 (26)	

Analysis were performed using R × C Chi-square test, or Kruskal-Wallis test.
*: P<0.05.
Abbreviations: AFP, alpha-fetoprotein; ALT, Alanine aminotransferase; γ-GT, gamma-glutamyl transpeptidase; HBsAg, hepatitis B surface antigen; TACE, transhepatic arterial chemotherapy and embolization.

Table 2
Univariate and multivariate analysis for early recurrence in patients with CTC ≥ 1

Variable	Training Cohort (n=63)						Validation cohort (n=126)					
	HR	95% CI			*P* value		HR	95% CI			*P* value	
Univariate Analysis												
Gender (Male)	0.61	0.27	-	1.41	0.251		0.91	0.44	-	1.91	0.807	
Age ≥ 60	0.76	0.36	-	1.59	0.468		0.74	0.43	-	1.26	0.271	
Largest diameter > 5 cm	2.84	1.40	-	5.75	0.004	*	3.07	1.90	-	4.95	<0.001	*
Multiple tumors	4.31	2.17	-	8.59	<0.001	*	2.94	1.82	-	4.76	<0.001	*
Incomplete tumor capsule	1.57	0.78	-	3.19	0.209		2.26	1.41	-	3.62	0.001	*
Edmondson grades III-IV	1.36	0.70	-	2.68	0.366		2.28	1.42	-	3.67	0.001	*
Vascular invasion	2.83	1.28	-	6.26	0.010	*	2.88	1.77	-	4.70	<0.001	*
Liver cirrhosis	0.83	0.42	-	1.63	0.595		1.59	0.98	-	2.58	0.060	
Positive HBsAg	2.15	0.66	-	7.04	0.206		1.78	0.65	-	4.90	0.261	
AFP > 400 ng/mL, n (%)	2.21	1.10	-	4.42	0.025	*	2.51	1.56	-	4.03	<0.001	*
Child-Pugh B class	1.28	0.17	-	9.34	0.811		1.00	0.00	-	4E9	1.000	
Surgical margin > 1 cm	0.22	0.07	-	0.71	0.011	*	0.40	0.22	-	0.75	0.004	*
Adjuvant TACE	1.73	0.88	-	3.41	0.114		0.85	0.50	-	1.43	0.534	
Multivariate Analysis (Backward Conditioned)												
Largest diameter > 5 cm	2.19	1.05	-	4.59	0.038	*	1.81	1.08	-	3.05	0.026	*
Multiple tumors	3.93	1.89	-	8.16	<0.001	*	2.28	1.36	-	3.82	0.002	*
Edmondson grades III-IV					NS		1.87	1.13	-	3.10	0.015	*
AFP > 400 ng/mL	2.91	1.41	-	6.03	0.004	*	2.34	1.43	-	3.82	0.001	*
Surgical margin > 1 cm	0.20	0.06	-	0.67	0.009	*	0.47	0.25	-	0.90	0.023	*

Analysis were performed using Cox regression test.
* : P<0.05.
Abbreviations: AFP, alpha-fetoprotein; HBsAg, hepatitis B surface antigen; NS, not significant; TACE, transhepatic arterial chemotherapy and embolization.

log-rank test, Figure S2A). The "X-tile" also indicated that surgical margin of >0.9 cm had the smallest *P* value. Thus, we chose a surgical margin of 1 cm as the optimal threshold for further analysis.

We found that CTC-positive patients with a surgical margin of >1 cm showed significantly longer TTER (not reached *vs.* 8.6 months) and lower early recurrence rates than those with a surgical margin of ≤1 cm (20.0% *vs.* 65.1%, *P*=0.005, log-rank test, Fig. 3A). Multivariate analysis confirmed that a surgical margin of >1 cm was an independent preventive factor for early recurrence in CTC-positive patients (HR, 0.20; 95% CI, 0.06-0.67; *P*=0.009, Cox regression test, Table 2). Moreover, patients with a surgical margin of >1 cm showed improved 5-year OS rates compared with those in the margin$^{\leq 1}$ group (79.0% *vs.* 52.1%, *P*=0.078, log-rank test, Fig. 3B), although statistical significance was not reached.

For the 54 patients in the training cohort with undetectable CTC, the margin$^{>1}$ group had similar TTER with those in the margin$^{\leq 0.5}$ (not reached *vs.* 14.2 months, *P*=0.348, log-rank test) and margin$^{0.5\text{-}1}$ (not reached for both, *P*=0.486, log-rank test) groups, and no significant difference in TTER was found between the margin$^{\leq 0.5}$ and margin$^{0.5\text{-}1}$ groups (*P*=0.144, log-rank test, Figure S2B). For preoperative CTC-negative patients, using a surgical margin of 0.5 cm did not impact TTER (*P*=0.148, log-rank test, Fig. 4A) and OS (*P*=0.113, log-rank test, Fig. 4B). And using a surgical margin of 1 cm impact neither TTER (*P*=0.767, log-rank test, Figure S3A) nor OS (*P*=0.113, log-rank test, Figure S3B).

In addition, we also found no significant difference between the effects of surgical margins of >2 cm and 1−2 cm on early recurrence in CTC-positive and CTC-negative patients, respectively (*P*=0.559 and *P*=0.812, log-rank test, Figure S4A-B). For late recurrence, no significant difference was observed between the margin$^{>1}$ and margin$^{\leq 1}$ groups in CTC-positive group (*P*=0.407, log-rank test, Figure S5A), and no significant difference was observed between the margin$^{>0.5}$ and margin$^{\leq 0.5}$ in CTC-negative group (*P*=0.285, log-rank test, Figure S5B).

3.3. Effects of surgical margin on early postoperative recurrence in the validation cohort

To further verify our findings, we enrolled another independent cohort of patients with larger samples (*n*=192) and longer follow-up time (median, 99.9 months; range, 15.4−110.3 months). In the validation cohort, 125 patients experienced recurrence, and early recurrence accounted for the majority (98/125, 78.4%) of cases. The respective 2-year and 5-year cumulative recurrence rates were 51.6% and 63.4%, and the 2-year and 5-year OS were 78.6% and 60.0%. No significant difference of baseline characteristics was found among different surgical margins (Table S2).

For the 126 CTC-positive patients in the validation cohort, the patients in margin$^{>1}$ group had significant longer TTER compared to those in the margin$^{\leq 1}$ group (not reached *vs.* 10.1 months, *P*=0.003, log-rank test, Fig. 3C), which was consistent with the results of the training cohort. The estimated rate of early recurrence was 36.4% and 65.1% in the margin$^{>1}$ and margin$^{\leq 1}$ groups, respectively. In multivariate analyses, a surgical margin of >1 cm was still an independent factor for early recurrence in CTC-positive patients (HR, 0.47; 95% CI, 0.25−0.90; *P*=0.023, Cox regression test, Table 2); other tumor-associated independent risk factors of early recurrence were large tumor diameter, multiple tumors, high Edmondson grade, and AFP >400 ηg/mL. Patients with a surgical margin of >1 cm survived longer than those with a surgical margin of ≤1 cm (OS median, not reached *vs.* 70.1 months, *P*=0.025, log-rank test, Fig. 3D). Regarding late recurrence, no significant difference was observed between the margin$^{>1}$ and margin$^{\leq 1}$ groups (*P*=0.875, log-rank test, Figure S5C).

For the 66 CTC-negative patients, surgical margin >0.5 did not influence TTER (*P*=0.813, log-rank test, Fig. 4C), OS (*P*=0.392, log-rank test, Fig. 4D), late recurrence (*P*=0.754, log-rank test, Figure S5D). Surgical margin >1 influenced neither TTER (*P*=0.186, log-rank test, Figure S3C) nor OS (*P*=0.092, log-rank test, Figure S3D).

3.4. Effects of surgical margin on postoperative liver function in the training and validation cohorts

To assess the effect of surgical margin on impairment and restoration of liver function, we compared changes of TB, ALT, AST, and PT levels in all patients (n=309, combing training and validation cohort) with different surgical margin sizes.

For CTC-positive patients (*n*=189), TB on POD 1, 3, 5, 7 were 26.4±9.9, 37.3±15.2, 31.5±14.9, 30.2±31.4 μmol/L in the margin$^{\leq 1}$ group and were 32.3±20.6, 38.6±20.9, 34.4±19.5, 31.3±32.1 μmol/L in

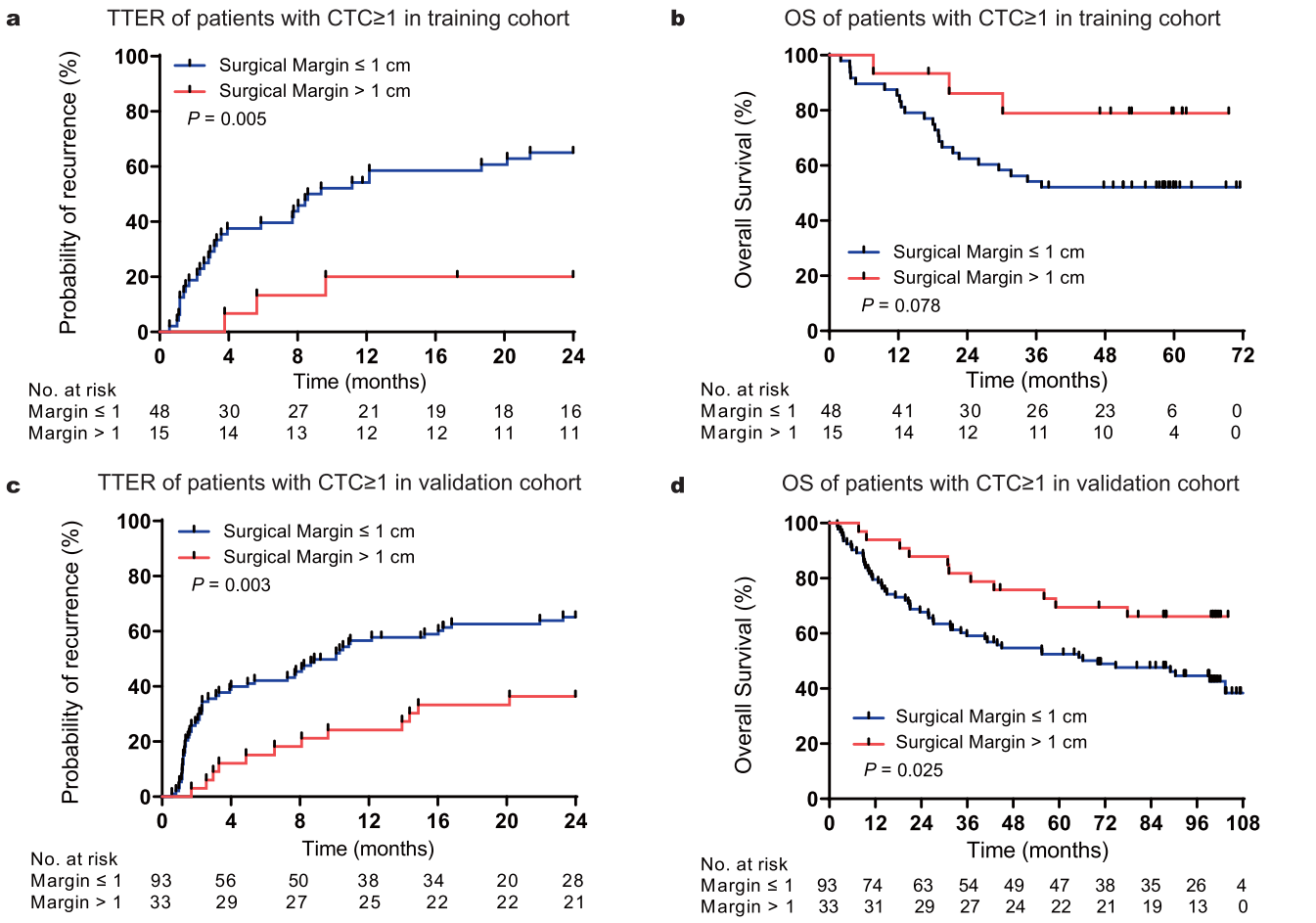

Fig. 3. Comparison of TTER and OS for CTC-positive patients. (A) TTER and (B) OS of CTC-positive patients between surgical margin of ≤1 cm and >1 cm in the training cohort. (C) TTER and (D) OS of CTC-positive patients between surgical margin of ≤1 cm and >1 cm in the validation cohort. (CTC, circulating tumor cell; OS, overall survival; TTER, time to early recurrence.) Log-rank test was used.

margin$^{>1}$ group, respectively (Figure S6A). ALT on POD 1, 3, 5, 7 were 517.1±434.1, 345.0±310.8, 188.8±134.2, 131.8±77.7 U/L in the margin$^{\leq 1}$ group and were 641.8±551.9, 352.8±268.8, 188.6±110.9, 151.1±59.2 U/L in the margin$^{>1}$ group, respectively (Figure S6B). AST on POD 1, 3, 5, 7 were 540.4±419.7, 133.5±131.5, 52.2±30.6, 54.3±40.6 U/L in the margin$^{\leq 1}$ group and were 577.8±491.2, 111.5±62.6, 45.1±16.9, 49.8±22.2 U/L in the margin$^{>1}$ group, respectively (Figure S6C). PT on POD 1, 3, 5, 7 were 14.0±1.4, 14.7±1.9, 14.0±1.1, 13.6±1.9 s in the margin$^{\leq 1}$ group, and were 14.1±1.5, 14.5±1.5, 14.3±1.4, 14.2±1.6 s in the margin$^{>1}$ group, respectively (Figure S6D). No significant difference was found in postoperative levels of TB ALT, AST, and PT between margin$^{\leq 1}$ and margin$^{>1}$ groups on POD 1, 3, 5 and 7 in CTC-positive patients. The incidence rate of Grade C PHLF in the margin$^{\leq 1}$ group and margin$^{>1}$ groups were 2.1% (3/141) and 4.2% (2/48). No significant difference was found between margin$^{\leq 1}$ and margin$^{>1}$ groups (P=0.811, chi-square test).

For CTC-negative patients (n=120), we also saw no significant difference in postoperative levels of TB, ALT, AST, and PT between margin$^{\leq 0.5}$ and margin$^{>0.5}$ groups on POD 1, 3, 5 and 7. And no difference was found in the incidence rate of Grade C PHLF between the margin$^{\leq 1}$ group and margin$^{>1}$ group [2.4% (1/42) *vs.* 0.0% (0/78), P=0.752, chi-square test].

4. Discussion

Despite undergoing curative resection, HCC patients still often experience a high rate of recurrence [36]. The early postoperative recurrence of HCC accounts for more than 70% recurrent or metastatic cases [37], and mVI is one of the most important recurrence-related risk factors [12,13,38]. Several studies have reported that a wide surgical margin may prevent early postoperative HCC recurrence in patients with mVI [7,39]; however, predicting mVI to establish a precise surgical excision range before hepatectomy remains challenging. We found that preoperative CTC status could identify HCC patients with high risk of mVI as well as its severity, as CTC status positively corelated with the number and distance of mVI in peritumoral tissues. More importantly, a surgical margin of >1 cm would benefit CTC-positive patients by eliminating mVI-associated MRD to prolong TTER and reduce early recurrence rates, while surgical margin minimally impacted early HCC recurrence in CTC-negative patients whenever using 0.5 cm or 1 cm as the threshold in this study. Thus, preoperative CTC status may contribute to the determination of optimal surgical margins to improve clinical outcomes for HCC patients undergoing resection.

Early recurrence typically results from MRD via HCC dissemination, which is undetectable before resection by conventional imaging [40,41]. Numerous studies have shown that CTC status could be a predictive marker for disease progression and correlates with an aggressive disease phenotype [42–44]. We previously demonstrated that preoperative CTC ≥2 correlated with early recurrence and vascular invasion in HCC patients [23]. In this study, we confirmed that the presence of CTC correlates with the number as well as dissemination distance of mVI, implying the risk of residual mVI after resection is high in CTC-positive patients. Although mVI was also found in CTC-negative patients, its number and distance were significantly less than those in CTC-positive patients. Thus, a wide surgical margin is

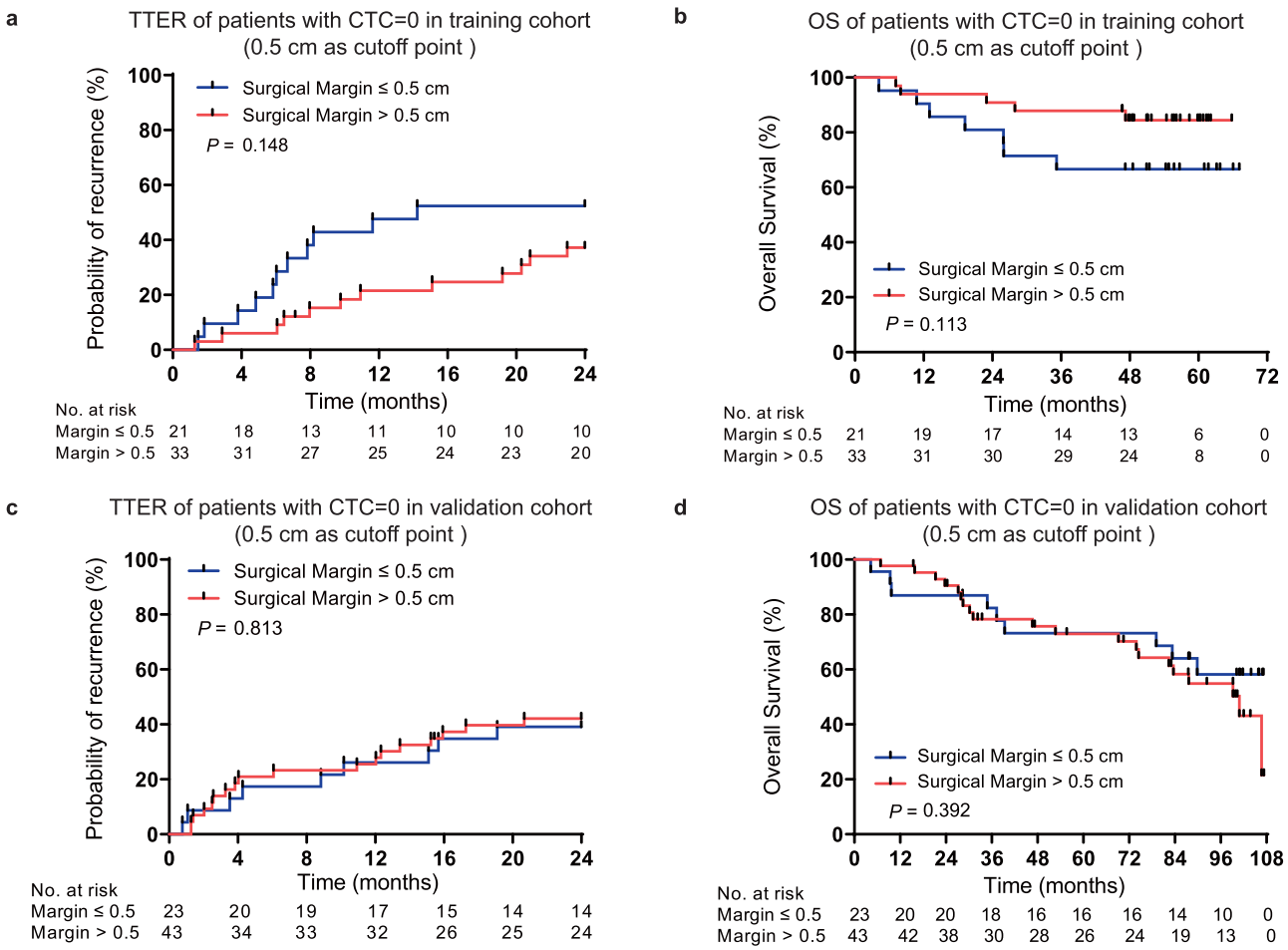

Fig. 4. Comparison of TTER and OS for CTC-negative patients. (A) TTER and (B) OS of CTC-positive patients between surgical margin of ≤0.5 cm and >0.5 cm in the training cohort. (C) TTER and (D) OS of CTC-positive patients between surgical margin of ≤0.5 cm and >0.5 cm in the validation cohort. (CTC, circulating tumor cell; OS, overall survival; TTER, time to early recurrence.) Log-rank test was used.

needed for patients with detected CTC to limit the recurrence and metastases caused by residual mVI in peritumoral tissues, while a narrow margin is adequate for patients without preoperative CTC.

The relationship between surgical margin and oncologic outcome is unclear in HCC, as there is no consensus for an adequate curative surgical margin [45–48]. Although achieving a sufficient margin from the tumor is the goal of oncologic surgery, preserving liver parenchyma to prevent postoperative liver failure is important for HCC patients, especially for those with chronic liver disease or cirrhosis. Thus, we performed a comprehensive study on mVI distribution according to preoperative CTC status and found that a wider resection margin >1 cm was optimal for CTC-positive patients, while a narrow resection margin of 0.5 cm was sufficient for patients without detectable CTC, especially in HCC patients with compromised liver function. Kaplan-Meier analyses revealed higher early recurrence rates in CTC-positive patients with a surgical margin ≤1 cm *vs.* >1 cm, while no significant improvement in early recurrence was seen with wider surgical margins (1-2 cm *vs.* >2 cm). Multivariate analyses confirmed that a surgical margin of >1 cm was an independent prognostic factor for early postoperative recurrence in these patients but not in CTC-negative patients. These results were validated in another independent cohorts (n=192) with a longer follow-up period.

The mVI is confirmed by microscopic examination of resected specimens, which cannot be achieved preoperatively. However, preoperative CTC could enable precise determination of surgical margin to guide treatment. Patients with preoperative CTC could experience lower rates of early recurrence with a surgical margin of >1 cm. Considering such surgical margins still does not guarantee eradication of all HCC in CTC-positive patients, and postoperative adjuvant therapy could help prevent early HCC recurrence after surgical resection. For patients without CTC, we hypothesized that they might safely undergo a resection of narrower margin without impaired prognosis. Although the resection margin of >0.5 failed to show better prognosis in both training and validation cohort, there remained a possibility that small sample size concealed the difference. The beneficial effect of surgical margin might be less obvious in CTC-negative than CTC-positive group.

The width of the resection margin did not significantly impact the frequency or severity of surgical complications or postoperative mortality. According to postoperative liver function tests, ALT, AST, TB, and PT reached their peaks between POD 1 and 3 and gradually returned to normal. No postoperative differences in the levels of any parameter were observed with respect to different surgical margins.

In this study, CTC was detected in 53.8% of patients in the training cohort and 65.6% of patients in the validation cohort. Although CTC has a rare frequency in the circulation, it could be detected in 100% of HCC patients by certain detection methods [49,50]. For EpCAM-based CellSearch system in HCC, the detection rate of CTC ranged from 15% to 67% [51]. The variation might be caused by the difference among baseline characteristics. From the view of inclusion criteria, the research focusing on the early HCC showed the lowest detection rate, and the studies recruiting advanced HCC reported higher detection rate. Specifically, previous reports and our data (Table S3) indicated

that positive CTC correlated with infection of HBV or HCV, larger diameter, vascular invasion, high level of AFP, and advanced stage of Barcelona Clinic Liver Cancer (BCLC) or China Liver Cancer (CNLC), all of which might impact detection rate [17,19,23,52].

Several limitations should be considered in interpreting our results. First, this investigation was a retrospective cohort study. Potential selection bias or confounding factors could not be eliminated. The conclusions drawn from this study should be verified in a larger prospective study. Second, the etiology of HBV infection accounted for the vast majority of patients in this study. It needs further validation whether the findings in this study applicable to HCC caused by HCV or NASH (non-alcoholic steatohepatitis). Third, defining mVI by histopathology is inherently limited because only seven regions in the same plane were examined for each specimen. Therefore, our mVI data might not fully represent its entire distribution landscape. Alternately, CTC as a "liquid biopsy", well-reflecting tumor biological phenotypes, may better reflect the degree of tumor dissemination. Fourth, we used the CellSearch® (the only one approved by FDA) to detect CTC in this study in view of its stability and relatively widely usage. Besides CellSearch® using ferrofluid beads functionalized with anti-EpCAM, there were various other platforms being developed to identify and capture CTC with high efficiency. Among them, karyoplasmic ratio [53] (enrichment-free method using imaging flow cytometry), CanPatrol [54] (RNA in suit hybridization of EMT markers after microfiltration), and Cyteel [55] (negative enrichment followed by centromere staining) had been used to predict vascular invasion with a good performance. All these methods have their advantages in some aspect. Further head to head comparative studies are needed to evaluate the prediction value of different detection methods.

In conclusion, preoperative CTC status correlated with and predicted mVI severity in HCC patients and is an important parameter for determining optimal surgical margin size for ensuring disease eradication and conserving functional liver parenchyma.

Declaration of Competing Interests

There are no conflicts of interest to declare.

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Contributors

KQ.Z., YF.S., and XR.Y. were responsible for conception and design; J.F. and XR.Y. were responsible for administrative support; JW.C., and W.G. were responsible for CTC testing; Y.J. and M.D. were responsible for mVI evaluation; KQ.Z., YF.S., PX.W., B.H., Y.Y. and JF.H. were responsible for collection and assembly of data; KQ. Z., YF.S. JW.C., J.Z., F.J, and XR.Y. were responsible for data analysis and interpretation; all the authors took part in writing or revising of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.103107.

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