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ORIGINAL ARTICLE

Circulating tumor cells are associated with poor outcomes in early-stage hepatocellular carcinoma: a prospective study

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Abstract

Background Previous studies evaluating association between circulating tumor cells (CTCs) and clinical outcomes in hepatocellular carcinoma (HCC) have shown inconsistent results due to suboptimal detection methods and patient heterogeneity.

Methods Patients undergoing surgery for early-stage HCC were prospectively enrolled. The CTC numbers were determined using a tapered slit platform, which detects CTCs based on the cell size and morphology. Survival and recurrence were evaluated, and Cox proportional hazards models were used to demonstrate the prognostic significance of CTC.

Results Of 105 patients, 25 had increased CTC numbers after surgery ($\Delta CTC > 0$, defined as positive) and a significantly higher level of recurrence ($p = 0.042$). A positive ΔCTC was seen to be an independent predictor of recurrence (hazard ratio 2.28), along with hepatitis B virus infection, alanine aminotransferase level, and the presence of satellite nodules (all $p < 0.05$). Subgroup analyses showed that a positive ΔCTC was associated with lower survival and higher recurrence among patients with low alpha-fetoprotein levels and cirrhosis (all $p < 0.05$).

Conclusion Calculation of ΔCTC based on the physical properties of the cells is predictive of recurrence in patients with early HCC undergoing surgery.

Keywords Circulating tumor cells · Nanomedicine · Hepatocellular carcinoma · Recurrence · Survival

Abbreviations

CTC	Circulating tumor cell
HCC	Hepatocellular carcinoma
AFP	Alpha-fetoprotein
EpCAM	Epithelial cell adhesion molecule
TSF	Tapered slit filter
CK	Cytokeratin
CD	Cluster of differentiation
OS	Overall survival
RFS	Recurrence-free survival
SD	Standard deviation
HBV	Hepatitis B virus
AST	Aspartate aminotransferase

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MELD	Model for end-stage liver disease
IQR	Interquartile range
CI	Confidence interval
HR	Hazard ratio
ALT	Alanine aminotransferase

Introduction

Circulating tumor cells (CTCs) detected in the peripheral blood of patients with solid tumors because malignant neoplasms invade blood vessels during metastatic colonization [1]. In hepatocellular carcinoma (HCC), a blood alpha-fetoprotein (AFP) mRNA was assessed as a marker of CTCs and was shown to be associated with early metastasis and poor survival [2]. However, while some studies have suggested that CTCs are associated with poor prognosis in HCC [3], others have demonstrated no correlation with clinical outcomes [4, 5].

Of note, the previous studies included heterogeneous populations comprising patients with early to advanced tumors [2, 4, 6]. It is not surprising that patients with advanced-stage cancer and high tumor burden release more CTCs in the blood. These patients are also more likely to have rapidly progressing disease and, therefore, poor prognosis [3, 7]. In addition, because HCC has molecular heterogeneity even within the same patient and CTC numbers are usually low, a biochemical method based on a limited number of molecules, such as epithelial cell adhesion molecule (EpCAM), may not have sufficient sensitivity to detect CTCs.

Therefore, we investigated the prognostic importance of CTCs in homogenous patients undergoing curative surgery for early-stage HCC. We particularly focused on the change in CTC numbers before and after surgery (ΔCTC), because even if the tumor burden is relatively small, the surgical procedure itself introduces the risk of tumor cells being shed into the bloodstream and potentially acting as a focus for recurrence and metastasis. In addition, to improve the sensitivity of CTC detection, a method that utilises differences in physical properties between CTCs and normal blood cells was used rather than traditional biochemical methods.

Materials and methods

Study design and participants

Between July 2014 and June 2016, patients diagnosed with early-stage HCC (Barcelona Clinic Liver Cancer stage 0 or A) and undergoing curative surgery at the Asan Medical Center were prospectively enrolled. A diagnosis of HCC was established according to the current guideline [8] and confirmed by postoperative pathologic findings. Patients were excluded if the final pathologic diagnosis was not HCC, if consent was withdrawn, or if another treatment other than surgery was performed after obtaining informed consent. A control group comprised patients with liver cirrhosis; the absence of HCC was confirmed by regular imaging surveillance at the Ewha Womans University Mokdong Hospital during the same study period.

Clinical and laboratory characteristics were collected in all participants and radiologic and pathologic tumor characteristics were identified in the HCC group. In HCC patients, imaging surveillance was performed using dynamic contrast-enhanced computed tomography 4–6 weeks after surgery and every 3 months thereafter.

The study design was approved by the institutional review board and the study was performed in accordance with the Declaration of Helsinki. All the participants provided written, informed consent.

Laboratory evaluation of CTCs

Blood samples were refrigerated and shipped in cold storage to the Korea Advanced Institute of Science and Technology (KAIST) within 6 h. CTCs were isolated and enumerated using a tapered slit filter (TSF) platform (Electronic Supplementary Material S1, [9]). The TSF platform consists of the following three layers: top chamber (16 × 12 mm), TSF (16 × 12 mm; effective area, 10 × 10 mm), and bottom chamber (16 × 12 mm). Blood samples are passed through from the top to the bottom chamber and CTCs are captured in the filter, which consists of 34,445 tapered slits that gradually narrow towards the exit. The platform was optimised using a numeral equation as described previously, and its capture efficiency was reported to be 89.87% [10].

Blood samples from each patient (5 mL) were diluted in phosphate-buffered saline solution (10 mL) and processed in the TSF platform using a syringe pump (Electronic Supplementary Material S2). The captured cells were released by applying a reverse flow of the solution and the released cells were mounted on a glass slide for immunofluorescence. Images were taken and quantified using MetaMorph® software (Molecular Devices, Sunnyvale, CA, USA). The cells were defined as CTCs when they met both the staining (positive for 4′,6-diamidino-2-phenylindole and cytokeratin (CK); negative for cluster of differentiation (CD) 45) and morphological (higher nucleus-to-cytoplasm ratio, higher degree of irregularity than background cells, larger size) criteria. ΔCTC was calculated by subtracting the number of preoperative CTCs from the postoperative CTCs; ΔCTC > 0 was defined as a positive ΔCTC.

Histologic examination of tumor tissues

The surgical specimens were examined by pathologists with ≥ 10 years of experience. A gross examination of the specimen was performed to determine any obvious areas of tumor remaining at the resection margin. Serial sections of the tumor and the surrounding tissues were evaluated to identify any microvascular invasion, Glisson capsule invasion, necrosis, dysplastic nodules, satellite nodules, and positive microscopic margins. The presence of cirrhosis was also reported.

Statistical analysis

Follow-up for HCC patients was completed in June 2018. Overall survival (OS) and recurrence-free survival (RFS) were estimated by Kaplan–Meier analysis and compared using the log-rank test. OS was defined as the time from the date of surgery to death from any cause; recurrence was determined as the time from surgery to the first radiologic demonstration of new HCC lesion(s). If death or recurrence did not occur during the study period, the data were censored on the date of last follow-up.

Associations between baseline characteristics and OS and RFS were determined using Cox proportional hazards models. Subgroup analyses were performed in patients with low alpha-fetoprotein (AFP) levels and those with liver cirrhosis.

Data are presented as mean ± standard deviation for continuous variables and as number and percentage for categorical variables. The relationship between ΔCTC and continuous variables was assessed using the Student's *t* test or Wilcoxon rank-sum test. Any relationship between ΔCTC and categorical variables was assessed using the Chi-square or Fisher's exact test. All reported *p* values are two-sided; *p* values of < 0.05 were considered statistically significant. All statistical analyses were performed using the SPSS statistical package (SPSS version 22.0 for Windows; SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

A total of 254 patients were screened (Electronic Supplementary Material S3). Of 121 HCC patients, eight patients whose final diagnosis was not HCC (six with combined HCC and cholangiocarcinoma and two with focal nodular hyperplasia), six who underwent transarterial chemoembolisation, one who was also diagnosed with another malignancy, and one who withdrew consent, were excluded. Finally, 105 HCC patients were analysed. The control group comprised of 133 patients with liver cirrhosis and no evidence of HCC. After excluding one patient who withdrew consent, 132 patients were analysed.

Blood was taken before (9.8 ± 8.5 days) and after (2.3 ± 5.7 days) surgery in the HCC group and at the time of outpatient visits in patients with liver cirrhosis. Evaluation of CTC numbers was technically possible in all patients. Representative images of CTCs, enumerated by the TSF platform, are provided in Electronic Supplementary Material S4a. Both EpCAM-positive and -negative CTCs were detected in one patient, whose surgical specimen showed a mixed histological phenotype of trabecular and scirrhous features (Electronic Supplementary Material S4b). Another example is the CTC clusters that were partially positive for EpCAM (Electronic Supplementary Material S5a) and diagnosed as scirrhous HCC after surgery (Electronic Supplementary Material S5b).

The baseline characteristics are summarised in Table 1. Among 132 patients in the control group, 101 (76.5%) were negative for CTC. Only 1 CTC was detected in 22 (16.7%) patients and 2 CTCs were enumerated in the blood of 8 (6.1%) patients. There was only 1 patient who had 3 CTCs in the control group.

In comparison with the control group, patients with HCC had a higher level of hepatitis B virus (HBV) infection (81.0% vs. 38.6%; $p < 0.001$). Albumin levels were significantly higher (3.8 ± 0.4 g/dL vs. 3.3 ± 0.8 g/dL; $p < 0.001$); aspartate aminotransferase (AST) and total bilirubin levels were lower in the HCC group (36.0 ± 19.3 IU/L vs. 61.1 ± 62.9 IU/L for AST; 0.6 ± 0.3 mg/dL vs. 2.3 ± 3.5 mg/dL for bilirubin; both $p < 0.001$). Accordingly, the Child–Pugh scores were significantly lower in HCC patients. The mean CTC number in 5 mL of peripheral blood was 1.8 ± 2.4 and 0.3 ± 0.6 in the HCC and liver cirrhosis groups, respectively ($p < 0.001$).

For patients with HCC, ΔCTC was calculated by subtracting the number of preoperative CTCs from postoperative CTCs. According to ΔCTC values, patients were divided into two groups: ΔCTC > 0 (positive ΔCTC; $n = 25$) or ΔCTC ≤ 0 (negative ΔCTC; $n = 80$). Patients with a positive ΔCTC had larger tumors (52.2 ± 36.8 mm vs. 36.4 ± 22.2; $p = 0.05$) and higher levels of des-gamma-carboxy-prothrombin (DCP) [median, 180.5, interquartile ranges (IQR), 39.3–1467.8 vs. 56.5, 25.8–251.8; $p = 0.022$] than the negative ΔCTC group; however, other characteristics were comparable (Table 2). Approximately half of the patients in both groups (52.0% in the positive ΔCTC and 43.8% in the negative ΔCTC group) showed liver cirrhosis on surgical specimens. All patients had R0 resection and the proportion of patients with microvascular invasion and satellite nodules did not differ between the groups.

Table 1 Baseline patient characteristics

Characteristics	HCC ($n=105$)	Liver cirrhosis ($n=132$)	p value
Demographic characteristics			
Age, years	55.9 ± 9.6	57.9 ± 10.3	0.14
Male	86 (81.9)	93 (71.0)	0.07
BMI (kg/m^2)	24.3 ± 2.6	23.0 ± 3.1	0.001
Aetiology of liver disease, *n* (%)			
Hepatitis B virus	85 (81.0)	51 (38.6)	< 0.001
Hepatitis C virus	7 (6.7)	7 (5.3)	0.79
Alcohol	7 (6.7)	90 (68.2)[a]	< 0.001
Others or unknown	6 (5.7)	8 (6.1)	1.00
Biochemical characteristics			
Albumin, g/dL	3.8 ± 0.4	3.3 ± 0.8	< 0.001
Creatinine, mg/dL	0.9 ± 0.4	0.9 ± 0.4	0.51
AST, IU/L	36.0 ± 19.3	61.1 ± 62.9	< 0.001
ALT, IU/L	37.2 ± 24.0	35.4 ± 29.2	0.62
Total bilirubin, mg/dL	0.6 ± 0.3	2.3 ± 3.5	< 0.001
Prothrombin time, INR	1.0 ± 0.1	2.2 ± 10.3	0.27
Alpha-fetoprotein, ng/mL	12.0 (4.1–211.3)	3.7 (2.6–5.9)	0.12
Liver function characteristics			
Child–Pugh score	5.3 ± 0.5	7.0 ± 2.1	< 0.001
MELD score	7.2 ± 1.7	11.3 ± 5.1	< 0.001
CTC number[b]	1.8 ± 2.4	0.3 ± 0.6	< 0.001
Postoperative CTC number	1.5 ± 5.0	–	–

Values are presented as mean ± standard deviations, *n* (%) or median with interquartile range

BMI body mass index, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *INR* international normalised ratio, *MELD* model for end-stage liver disease, *CTC* circulating tumor cell

[a]21, 3, and 1 patient(s) also had hepatitis B virus infection, hepatitis C infection, and other liver disease, respectively

[b]Preoperative CTC number in HCC patients

Clinical outcomes

The median postoperative follow-up time was 31.1 months (IQR 26.7–35.0). The survival curve was dichotomised according to ΔCTC status and the mean OS time was longer in the negative ΔCTC group (40.8 months; 95% confidence interval [CI] 39.8–41.9) than in the positive ΔCTC group (38.9 months; 95% CI 35.6–42.2); however, the difference was not statistically significant ($p=0.19$; Fig. 1a). In terms of RFS, the curve showed significant separation according to ΔCTC status (mean RFS [95% CI] 33.4 [30.6–36.1] vs. 26.9 [20.6–33.2] months; $p=0.042$; Fig. 1b).

The Cox proportional hazards model showed that age and the presence of satellite nodules were associated with OS in univariate analysis. Multivariate analysis showed that only the presence of satellite nodules was associated with OS (hazard ratio [HR], 41.66; 95% CI 4.26–407.18; Table 3). With regard to RFS, the presence of HBV, tumor size, AST levels, alanine aminotransferase (ALT) levels, presence of satellite nodules, and positive ΔCTC were associated with recurrence in the univariate analysis (Table 4). On multivariate analysis, the presence of HBV (HR [95% CI] 0.37 [0.17–0.84]; $p=0.017$), ALT levels (1.01 [1.00–1.03]; $p=0.019$), presence of satellite nodules (9.34 [2.62–33.32]; $p=0.001$), and positive ΔCTC (2.28 [1.06–4.90]; $p=0.036$) were associated with RFS.

Subgroup analyses

To determine whether ΔCTC could be utilised in patients with low levels of tumor marker, subgroup analysis was performed in patients whose AFP levels were < 200 ng/mL ($n=79$). OS was significantly shorter in the positive ΔCTC group (mean [95% CI] 37.5 [32.9–42.1] vs. 41.2 [40.5–41.9] months; $p=0.047$) (Fig. 2a). RFS was also shorter in the positive ΔCTC group (mean [95% CI], 18.9 [13.3–24.6] vs. 35.4 [32.8–38.0] months; $p<0.001$) (Fig. 2b).

Table 2 Baseline characteristics of patients who underwent curative surgery for early HCC

Variables	ΔCTC > 0 ($n = 25$)	ΔCTC ≤ 0 ($n = 80$)	p value
Demographic characteristics			
Age, years	56.6 ± 8.8	55.7 ± 9.9	0.67
Male	22 (88.0)	64 (80.0)	0.55
BMI, kg/m^2	24.8 ± 2.2	24.2 ± 2.7	0.34
Aetiology of liver disease			
Hepatitis B virus	19 (76.0)	66 (82.5)	0.56
Hepatitis C virus	0 (0.0)	7 (8.8)	0.19
Alcohol	3 (12.0)	4 (5.0)	0.35
Others or unknown	3 (12.0)	3 (3.8)	0.15
Tumor characteristics			
Size, mm	52.2 ± 36.8	36.4 ± 22.2	0.05
Multiple numbers	3 (12.0)	6 (7.5)	0.44
BCLC stage			
0	2 (8.0)	18 (22.5)	0.15
A	23 (92.0)	62 (77.5)	
Biochemical characteristics			
Albumin, g/dL	3.7 ± 0.4	3.9 ± 0.4	0.14
Creatinine, mg/dL	0.9 ± 0.1	0.9 ± 0.5	0.77
AST, IU/L	38.4 ± 21.5	35.2 ± 18.7	0.47
ALT, IU/L	37.3 ± 19.4	37.2 ± 25.4	0.98
Alkaline phosphatase, IU/L	87.8 ± 32.2	78.0 ± 47.6	0.34
GGT, IU/L	81.8 ± 72.9	64.0 ± 84.2	0.35
Total bilirubin, mg/dL	0.6 ± 0.2	0.6 ± 0.3	0.76
Prothrombin time, INR	1.0 ± 0.1	1.0 ± 0.1	0.35
Alpha-fetoprotein, ng/mL	15.8 (4.9, 399.9)	10.7 (3.9, 121.8)	0.17
DCP, mAU/mL	180.5 (39.3, 1467.8)	56.5 (25.8, 251.8)	0.022
Liver function characteristics			
Presence of cirrhosis	13 (52.0)	35 (43.8)	0.50
Child–Pugh score	5.4 ± 0.6	5.2 ± 0.4	0.08
MELD score	7.1 ± 0.8	7.3 ± 1.9	0.75
Pathological characteristics			
Microvascular invasion	9 (36.0)	21 (26.3)	0.45
Satellite nodules	2 (8.0)	2 (2.5)	0.24
Histologic subtypes			
Trabecular type	22 (88.0)	76 (95.0)	0.35
Pseudoglandular type	8 (32.0)	27 (33.8)	1.00
Compact type	1 (4.0)	4 (5.0)	1.00
Scirrhous type	3 (12.0)	3 (3.8)	0.15
Edmondson–Steiner grade, III–IV	20 (80.0)	58 (72.5)	0.81

Values are presented as mean ± standard deviation, *n* (%), or median with interquartile ranges

HCC hepatocellular carcinoma, *CTC* circulating tumor cells, *BMI* body mass index, *BCLC* Barcelona Clinic Liver Cancer, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma-glutamyltranspeptidase, *INR* international normalised ratio, *DCP* des-gamma-carboxy-prothrombin, *MELD* model for end-stage liver disease

Subsequently, a total of 48 patients with confirmed liver cirrhosis were evaluated. The 3 year OS rate was significantly higher in patients with a negative ΔCTC (Fig. 2c; 100.0% vs. 84.6%; $p = 0.013$). Similarly, patients with a negative ΔCTC showed 100.0% 3 year RFS vs. 84.5% in the positive group (Fig. 2d, $p = 0.006$).

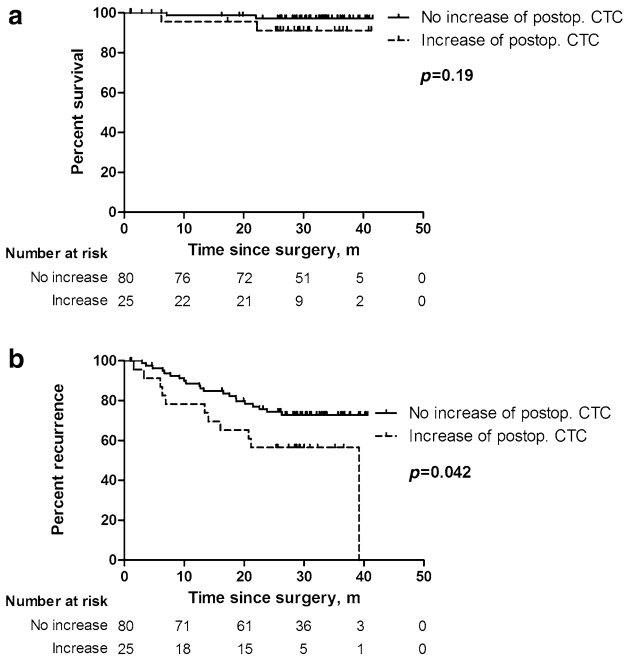

Fig. 1 Kaplan–Meier curve for **a** OS and **b** RFS in all patients

Clinical features of patients with increased CTC after surgery

Thirteen out of 25 positive ΔCTC patients (52.0%) showed the increase of postoperative CTC count ≥ 2 (range 2–47). The clinical characteristics of these patients are shown in Electronic Supplementary Material S6.

Of these 13 patients, 7 (53.8%) experienced recurrence, which was confined to the liver in 6 patients. The remaining one patient experienced recurrence as lung metastasis. A total of 10 patients (84.6%) had chronic HBV infection. Cirrhosis was identified in 9 patients (69.2%) on examination of the surgical specimen. With regard to tumor markers, AFP was < 50 ng/mL in 84.6% of the patients (median 9.6; IQR 3.7–17.1), whereas DCP was higher than 90 mAU/mL in 8 of 12 patients (66.7%; one patient had a missing value).

Discussion

This prospective study demonstrates that CTCs can be detected in early-stage HCC using a method based on the physical properties of the cells. The increased CTC counts after surgery (positive ΔCTC) were an independent predictor for recurrence. ΔCTC was significantly associated with OS

Table 3 Hazard ratios of baseline characteristics associated with overall survival

Variable	Univariable analysis		Multivariable analysis	
	Hazard ratio (95% confidence interval)	*p* value	Hazard ratio (95% confidence interval)	*p* value
Age, per 1 year	1.13 (1.00–1.28)	0.049	1.18 (1.00–1.40)	0.05
Male	0.67 (0.07–6.41)	0.73		
BMI, per 1 kg/m^2	1.06 (0.73–1.53)	0.76		
Presence of HBV	0.19 (0.03–1.35)	0.10		
Tumor size, per 1 cm	1.08 (0.81–1.44)	0.62		
Tumor number, multiple	4.42 (0.46–42.54)	0.20		
AST, per 1 IU/L	0.99 (0.94–1.05)	0.82		
ALT, per 1 IU/L	1.00 (0.96–1.05)	0.91		
ALP, per 1 IU/L	1.00 (0.99–1.02)	0.79		
GGT, per 1 IU/L	1.00 (0.98–1.02)	0.77		
AFP, > 200 ng/mL	1.20 (0.12–11.49)	0.88		
DCP, > 90 mAU/mL	1.35 (0.19–9.58)	0.77		
Presence of cirrhosis	1.25 (0.18–8.88)	0.82		
Child–Pugh score, per 1 point	2.74 (0.54–14.05)	0.23		
MELD score, per 1 point	0.99 (0.45–2.19)	0.98		
Microvascular invasion	2.41 (0.34–17.10)	0.38		
Satellite nodules	31.98 (4.45–229.77)	0.001	41.66 (4.26-407.18)	0.001
Positive ΔCTC	3.43 (0.48–24.32)	0.22		
Detection of preoperative CTC	1.94 (0.20–18.64)	0.57		
Detection of postoperative CTC	4.41 (0.46–42.45)	0.20		

BMI body mass index, *HBV* hepatitis B virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *GGT* gamma-glutamyltranspeptidase, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy-prothrombin, *MELD* model for end-stage liver disease, *CTC* circulating tumor cell

Table 4 Hazard ratios of baseline characteristics associated with recurrence

Variable	Univariable analysis		Multivariable analysis	
	Hazard ratio (95% confidence interval)	*p* value	Hazard ratio (95% confidence interval)	*p* value
Age, per 1 year	1.03 (0.99–1.07)	0.12		
Male	1.52 (0.53–4.35)	0.43		
BMI, per 1 kg/m^2	1.02 (0.89–1.17)	0.76		
Presence of HBV	0.29 (0.14–0.60)	0.001	0.37 (0.17–0.84)	0.017
Tumor size, per 1 cm	1.17 (1.05–1.30)	0.006	1.08 (0.95–1.24)	0.24
Tumor number, multiple	1.48 (0.45-4.86)	0.52		
AST, per 1 IU/L	1.02 (1.00–1.03)	0.022	1.01 (0.99–1.03)	0.60
ALT, per 1 IU/L	1.02 (1.01–1.03)	0.003	1.01 (1.00–1.03)	0.019
ALP, per 1 IU/L	1.00 (0.99–1.01)	0.91		
GGT, per 1 IU/L	1.00 (1.00–1.01)	0.30		
AFP, > 200 ng/mL	1.22 (0.55–2.73)	0.63		
DCP, > 90 mAU/mL	2.04 (0.99–4.21)	0.06		
Presence of cirrhosis	1.69 (0.84–3.40)	0.14		
Child–Pugh score, per 1 point	2.03 (0.98–4.21)	0.06		
MELD score, per 1 point	1.03 (0.84–1.26)	0.77		
Microvascular invasion	1.15 (0.54–2.43)	0.71		
Satellite nodules	6.68 (2.02–22.17)	0.002	9.34 (2.62–33.32)	0.001
Positive ΔCTC	2.10 (1.01–4.35)	0.047	2.28 (1.06–4.90)	0.036
Detection of preoperative CTC	0.96 (0.47–1.95)	0.92		
Detection of postoperative CTC	1.83 (0.91–3.66)	0.09		

BMI body mass index, HBV hepatitis B virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *GGT* gamma-glutamyltranspeptidase, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy-prothrombin, *MELD* model for end-stage liver disease, *CTC* circulating tumor cell

and RFS in a subgroup of patients with low AFP levels and those with liver cirrhosis.

During invasion to local tissues, blood vessels, and distant organs, tumor cells enter the circulation and can be detected in the peripheral blood. Previous studies have used 'biochemical' markers to identify CTCs, which can be broadly categorised as 'epithelial (such as EpCAM)' or 'tumor-specific (such as AFP)'. It is undoubted that patients with progressive disease have larger tumor burden and resultantly, release more CTCs into the blood, and show poorer prognosis [3, 7, 11]. Therefore, whether the presence of CTC really has impact on the clinical outcomes should be evaluated in the homogeneous patient population, e.g., those with the same stage. In addition, detection techniques that use particular biochemical markers lack sensitivity, as tumor cells do not universally express specific antigens. Particularly, the expression of EpCAM can be lost during epithelial-mesenchymal transition in aggressive tumors; therefore, an EpCAM-based technique might not be able to detect CTCs in these tumors, where grave prognosis is expected [12]. Therefore, we used a physical method to detect CTCs in a homogenous patient population with early-stage HCC, accurately calculated ΔCTC, and demonstrated that a positive ΔCTC was a good independent predictor of recurrence.

Differences in the physical properties of cancer cells and normal blood cells, such as size, deformability, density, and electrical signature, have been used to detect CTCs by physical methods [13, 14]. When the blood passes through a specifically designed filter, CTCs larger and stiffer than the normal blood cells are captured in the filter. The filter can be assembled in a microfluidic device or in a macro-scale cassette in the form of a membrane. Although the detection efficiency and purity of CTCs by microfluidic device are > 90% [15], most microfluidics-based platforms consist of complicated components such as pressure generator and pneumatic tubes [16]. In addition, the lifespan of the device is relatively short because of material degradation, clotting, and fouling problems and the processing time for each sample is generally much longer [17]. By contrast, a cassette-based membrane filter, such as ours, is a simple and easy-to-use, penny-sized portable device that does not require any special instruments or resources, except for the syringe and syringe pump. It also enables rapid sample processing by increasing the flow rate, up to 40 mL of whole blood per hour, thereby being advantageous for use in clinical practice. In addition, the tapered slits used in our platform allow more efficient and reliable cell isolation than straight slits [10]. Finally, the captured cells are examined and reliably confirmed as CTCs

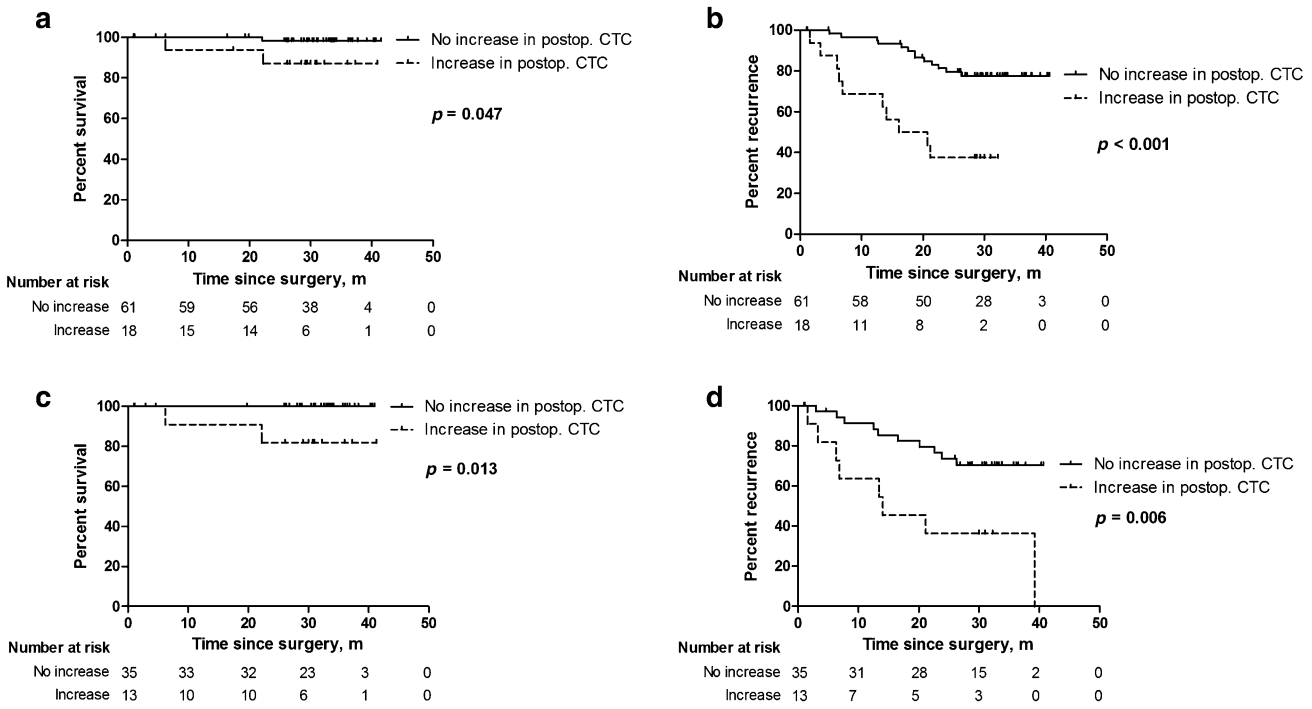

Fig. 2 **a** Kaplan–Meier curve for OS in patients with low alpha-fetoprotein levels (< 200 ng/mL); **b** Kaplan–Meier curve for RFS in patients with low alpha-fetoprotein levels (< 200 ng/mL); **c** Kaplan–Meier curve for OS in patients with liver cirrhosis; and **d** Kaplan–Meier curve for RFS in patients with liver cirrhosis

by immunofluorescence staining (CK-positivity and CD45-negativity) as well as by morphological features.

A significant number of preoperative CTCs and the cancer cells released into systemic circulation during the surgical procedure can undergo apoptosis [13, 18–20]. Positive ΔCTC, a simple and intuitive parameter that we used in this study, represents the CTCs that eventually survived after surgery. Some of these cells, if not all, might have metastatic potential resulting in poor prognosis, such as shorter RFS. Although the difference in OS time was not statistically significant according to ΔCTC status in this study, a positive ΔCTC increased the risk of death approximately threefold (HR, 3.43; 95% CI 0.48–24.32).

The predictive ability of ΔCTC was also observed in patients with low AFP levels and in those with liver cirrhosis. AFP levels are occasionally elevated in patients with active hepatitis or cirrhosis and could be challenging to interpret. In the current study, the mean CTC number was significantly lower in cirrhotic patients without HCC. In cirrhotic patients with HCC, ΔCTC status predicted both OS and RFS and could, therefore, be potentially useful in diagnosis and treatment of those patients.

We assessed postoperative CTCs at a relatively earlier timepoint (mean sampling day after surgery, 2.3 days) than in the previous studies including patients with other solid organ malignancies [21–23]. However, studies that included HCC patients counted CTCs at earlier postoperative period, ranging from the 3rd to the 9th days of surgery [24–26]. In particular, one of these studies, which evaluated CTCs immediately after surgery as well as at days 3 and 7, demonstrated that there is no significant difference in the CTC numbers between each timepoint [25]. In addition, Qi et al. identified that postoperative CTCs were associated with early recurrence, which they defined as recurrence within 6 months of surgery [26]. Indeed, postoperative recurrence occurred as early as 1.5 months after surgery in our data set. Therefore, considering the above studies that enumerated CTCs at an earlier postoperative period and the result that showed no significant difference in CTC numbers at several timepoints (immediate vs. day 3 vs. day 7), as well as the possibility of very early recurrence in HCC, we assessed postoperative CTCs before 1 week of surgery on an average. This earlier assessment can facilitate prompt prognostication, individualised planning for treatment and follow-up, and relevant education of patients, while they are in the hospital for postoperative care.

Certain limitations of this study must be also acknowledged. First, although our TSF platform has previously been validated and shown to have high detection ability (capture efficiency of 89.87%) [10, 27], it is theoretically possible that CTCs smaller than the slits or with high deformability might not be captured in the filter. However, considering that

the size of cancer cells, including those of HCC, is mostly between 10 and 20 μm according to the previous reports [9, 28, 29], we believe that our technology can be utilised for detecting CTCs in cancer patients. Second, CTCs were detected in the blood of patients from control group, i.e., those with liver cirrhosis but without HCC. We did not perform further experiments to characterise CK+/CD45− cells detected in control patients, as it was out of the scope of the current study. Notably, the most widely used EpCAM-based test for CTC detection, Cell Search®, also showed CTC positivity in 5% of healthy controls with or without benign tumors [30]. In addition, the previous studies that included benign tumors of the breast, colon, and pancreas also identified CTCs, although the average number was smaller than that in patients with cancer, as in our analysis (average number [SD], 0.3 [0.6] in liver cirrhosis vs. 1.8 [2.4] in HCC) [31–34]. Indeed, no test has been shown to achieve 100% sensitivity and specificity. In future studies, we hope to perform in-depth molecular analysis of CTCs that are detected in non-HCC patients, as well as determine false positive rate, and optimal cut-off point for CTC positivity.

In conclusion, we have identified CTCs in early-stage HCC using a novel technique based on the physical properties of the cells. The change in CTC numbers before and after surgery was an independent predictor of RFS. Further investigation in larger cohorts is required to confirm the value of this approach.

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Compliance with ethical standards

Conflict of interest Yeonjung Ha, Tae Hun Kim, Jae Eul Shim, Sunghyun Yoon, Mi Jung Jun, Young-Ho Cho, Han Chu Lee declare no conflicts of interest.

Informed consent in studies with human subjects The study design was approved by the institutional review board of the Asan Medical Center (Approval Number: 20140766), the Ewha Womans University Mokdong Hospital (Approval Number: 2014-08-004), and the Korea Advanced Institute of Science and Technology (Approval Number: KH2012-02). The study was performed in accordance with the Declaration of Helsinki. All the participants provided written informed consent.

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