Original Article

Circulating Cancer Stem Cells Expressing EpCAM/CD90 in Hepatocellular Carcinoma: A Pilot Study for Predicting Tumor Recurrence after Living Donor Liver Transplantation

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Background/Aims: Circulating tumor cells (CTCs) with cancer stemness have been demonstrated to be a direct cause of tumor recurrence, and only few studies have reported the role of CTCs in liver transplantation (LT) for hepatocellular carcinoma (HCC).

Methods: Epithelial cell adhesion molecule+ (EpCAM+), cluster of differentiation 90+ (CD90+) and EpCAM+/CD90+ CTCs were sorted via fluorescence-activated cell sorting, and transcripts level of *EpCAM, K19* and *CD90* in the peripheral blood were analyzed via real-time polymerase chain reaction preoperatively and on postoperative days 1 and 7 in 25 patients who underwent living donor liver transplantation (LDLT) for HCC. EpCAM protein was assessed in HCC tissue using immunohistochemical staining. The median follow-up duration was 40 months.

Results: HCC after LDLT recurred in four out of 25 patients. Detection of EpCAM+ or CD90+ CTCs correlated well with their messenger RNA levels ($p<0.05$). EpCAM+ CTCs were readily detected in HCC tissue expressing EpCAM protein. The detection of EpCAM+ CTCs or EpCAM+/CD90+ CTCs before surgery and on postoperative day 1 was significantly associated with HCC recurrence after LT (all $p<0.05$). Pretransplant serum PIVKA-II >100 mAU/mL and postoperative day 1 EpCAM+/CD90+ CTCs were independent risk factors for HCC recurrence (hazard ratio, 14.64; 95% confidence interval, 1.08 to 198.20; $p=0.043$ and hazard ratio, 26.88; 95% confidence interval, 1.86 to 387.51; $p=0.016$, respectively).

Conclusions: EpCAM+/CD90+ CTCs can be used preoperatively and 1 day after LDLT as key biological markers in LT candidate selection and post-LDLT management. **(Gut Liver 2022;16:443-455)**

Key Words: Circulating tumor cells; Hepatocellular carcinoma; Liver transplantation

INTRODUCTION

Liver transplantation (LT) has been the most effective treatment for multicentric, unresectable hepatocellular carcinoma (HCC) since the introduction of the Milan criteria.[1] However, the Milan criteria, which define macromorphological characteristics of HCC, still implies the risk of HCC recurrence in 10% to 15% of patients within the Milan criteria. As a result, high-risk patients within the Mi-

lan criteria will receive LT, but those who exceed the Milan criteria and have a low risk of recurrence are excluded from LT candidates. To overcome this limitation, the selection of LT has evolved into Metroticket 2.0 by adding the level of alpha-fetoprotein (AFP) to tumor size and tumor numbers, and further exploring several molecular markers, which can more directly represent tumor biology.[2] However, given the fact that LT can completely remove the diseased liver as well as tumor lesions, postoperative HCC

recurrence must be attributed to extrahepatic circulating tumor cells (CTCs), which can disseminate before LT or during surgery. Multiple studies have shown that CTCs are responsible for distant metastasis and postoperative recurrence in many cancers.[3-5] Recently, a small subset of CTCs with features of stem cells were found to be an actual founder that can contribute to metastasis and recurrence after treatment, in conjunction with the concept of cancer stem cells in tumor tissues.[6]

In HCC, side populations such as cluster of differentiation (CD) 133, keratin 19 (K19), CD90, oval cell marker 6, CD13, CD44, and epithelial cell adhesion molecule (EpCAM) have been demonstrated to have characteristics of cancer stem cells.[7,8] Among these markers, CD90 and EpCAM were used to detect CTCs with cancer stemness, and were found to show a direct correlation between the numbers of CTCs and HCC recurrence after curative liver resection.[9,10] We also reported that K19 expression in tumor tissue was more related to tumor aggressiveness and poorer prognosis after surgical resection[11] and the higher ratio of messenger RNA (mRNA) expression of *K19* and *CD44* in peripheral blood during the perioperative period was associated with early recurrence after curative resection. Even though analysis of CTCs with cancer stemness is an appealing method to predict HCC recurrence after LT, only a few studies have addressed this issue so far, and only one study has shown a positive correlation with recurrence after LT.[12-14]

EpCAM and CD90 were selected as representative factors of CTCs in this study because EpCAM is an epithelial stem cell marker and CD90 is a mesenchymal stem cell marker that is expressed on the cell surface. Therefore, fluorescence-activated cell sorting (FACs) of these markers was possible. The presence of EpCAM in tumor cells was associated with high serum AFP levels and poorly differentiated morphology of cancer cells, whereas the presence of CD90, which involves cell-to-cell and cell-to-matrix interactions, indicates that when CD90 is overexpressed in tumors, it promotes cancer dissemination and is associated with a high incidence of distant organ metastasis.[15,16]

In this study, we investigated the role of CTCs in cancer stemness-related genes and their mRNA levels in peripheral blood during the perioperative period to predict HCC recurrence in patients who received living donor liver transplantation (LDLT) for HCC.

MATERIALS AND METHODS

1. Patient selection and data collection

Between April 2014 and March 2017, we prospectively enrolled 25 patients who underwent LDLT for HCC at Severance Hospital, Seoul, Korea. All patients were diagnosed as having HCC via preoperative dynamic liver computed tomography and magnetic resonance imaging. Blood was obtained before starting surgery and at postoperative days 1 and 7. At each time point, 30 mL of peripheral blood was collected into a BD vacutainer containing K_2-EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) for FACs and real-time reverse transcription polymerase chain reaction (RT-qPCR). Five patients presented total tumor necrosis due to preoperative treatments (Table 1), and 20 HCC tissues were examined via immunohistochemical staining (Supplementary Material). All patients were followed up after discharge according to the protocols of our institute. This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (IRB number: 4-2012-0433). Written informed consent was obtained.

Histopathologic analysis was performed on all resected specimens by routine pathologic examination. The variables included tumor markers, tumor size, differentiation according to Edmondson-Steiner grade, multiplicity of tumors, microvascular invasion (MVI), and pathology details of non-tumor liver tissue were obtained from a review of medical records.

Patients were 38 to 65 years of age (median age 56) and consisted of 23 males and two females. The median follow-up time after transplantation was 39.50 months (range, 7.37 to 59.80 months). Twenty-two cases were hepatitis B virus-related, and the remainder were hepatitis C virus-related (n=1) or non-B, non-C hepatitis (n=2). Twenty patients received preoperative treatments. Twelve patients (48%) were initially above the Milan criteria. Of the 25 patients, five patients had undergone successful locoregional therapy to be downstaged within the Milan criteria before LDLT.

HCC recurrence after LDLT was observed in four patients (16%) during follow-up, a median of 38.63 months (range, 1.6 to 59.8 months), and two out of four patients received calcineurin inhibitor (CNI) with mammalian target of rapamycin inhibitor (mTORi), and the remaining patients were prescribed CNI with mycophenolate mofetil (MMF). Sorafenib was administered to all four patients with recurrence, of whom three patients underwent either metastatectomy or other locoregional therapy. Two patients started taking sorafenib after adrenalectomy and transarterial chemoembolization, respectively and showed stable disease until the last follow-up. One patient presented progress disease after combined treatments of sorafenib and locoregional treatment and the last patient was transferred to hospice care center after treated with sorafenib because of multiple lung, distal common bile duct, and

Table 1. Patient Baseline Clinicopathological Characteristics

Characteristics	Total (n=25)
Age, median (range), yr	56 (38–65)
Sex	
Male	23 (92)
Female	2 (8)
Etiology	
HBV	22 (88)
HCV	1 (4)
NBNC	2 (8)
Child-Pugh class at LT	
A	14 (56)
B	6 (24)
C	5 (20)
MELD scores at LT, median (range)	8 (6–25)
Pretransplant serum AFP	
<400 ng/mL	24 (96)
≥400 ng/mL	1 (4)
Pretransplant serum PIVKA-II	
<100 mAU/mL	17 (68)
≥100 mAU/mL	8 (32)
Cirrhosis	
Yes	20 (80)
No	5 (20)
Milan criteria	
Within	18 (72)
Beyond	7 (28)
BCLC staging system at LT	
0	4 (16)
A	8 (32)
B	9 (36)
C	4 (16)
UNOS staging classification at LT	
I	13 (52)
II	5 (20)
III	4 (16)
IV	3 (12)
Pretransplant treatment	
Liver resection	5 (20)
TACE	11 (44)
RFA	1 (4)
Radioembolization	1 (4)
CCRT	2 (8)
No pretransplant treatment	5 (20)
MVI	
Yes	6 (24)
No	19 (76)
Edmondson-Steiner grade	
I-II	12 (48)
III-IV	9 (36)
NA	4 (16)
Post-transplant usage of mTORi	
Yes	16 (74)
No	9 (36)
Follow-up period, median (range), mo	39.50 (7.37–59.80)

Data are presented as the number (%) unless otherwise indicated.
HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B, non-C hepatitis; LT, liver transplantation; MELD, Model for End-Stage Liver Disease; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence-II; BCLC, Barcelona Clinic Liver Cancer; UNOS, United Network for Organ Sharing; TACE, transarterial chemoembolization; RFA, radiofrequency ablation; CCRT, concurrent chemoradiotherapy; MVI, microvascular invasion; NA, not available; mTORi, mammalian target of rapamycin inhibitor.

bone metastasis without further evaluation.

2. Immunosuppressive agents

Cumulative dosages of immunosuppressant drugs were administered after transplantation based on the association of a CNI (tacrolimus or Prograf) and corticosteroids according to the immunosuppressant protocol employed at our hospital. Of the 25 patients, 13 patients were switched from MMF to everolimus within one month after surgery. In five patients, MMF was slowly changed to everolimus according to patient condition and the presence of biliary complications. Six patients took MMF and one patient did not use MMF or mTORi. Of the four recurrent patients, two were taking mTORi before HCC recurrence and continued to take it even after relapse. In the other two patients, MMF was switched to mTORi after HCC recurrence. Corticosteroid was added to immunosuppressant drugs for all 24 patients except one.

3. Flow cytometry

As mononuclear cells and CTCs were located in the same layer, mononuclear cells and CTCs were isolated from the 10 mL blood sample using density gradient centrifugation before FACs analysis. Briefly, 10 mL of blood was diluted with an equal volume of phosphate-buffered saline solution (Gibco, Grand Island, NY, USA) and layered onto Ficoll-Paque™ PLUS (GE Healthcare Bioscience, Pittsburgh, PA, USA) and centrifuged at 2,000 ×g for 20 minutes at 25℃. Thereafter, the layer of mononuclear cells was transferred to a new tube and washed.

The isolated cells were labeled with mouse-anti-human CD45-FITC, CD326 (EpCAM)-APC, and CD90-PE antibodies (Miltenyi Biotech Inc., Auburn, CA, USA). A combination of CD45– and CD90+ was used to define nonlymphatic CD90+ cells in the circulation. Labeled cells were detected via FACs using FACSCalibur (Becton Dickson Immunocytometry Systems, San Jose, CA, USA). Suitable isotypes of unrelated antibodies were used as negative controls. Blood specimens were processed approximately 2 hours after collection. The first gating was in the large cell region. After excluding CD45+ cells, CD45–/CD326+/CD90–, CD45–/CD326–/CD90+, and CD45–/CD326+/CD90+ cells were identified and counted in the CD45– population (Fig. 1).

4. Statistical analysis

All continuous data are reported as medians (range). The Mann-Whitney U test was used to compare continuous data, and the Fisher exact test was used to analyze categorical variables. The Pearson correlation coefficient was used to compare the amounts of CTCs and mRNA
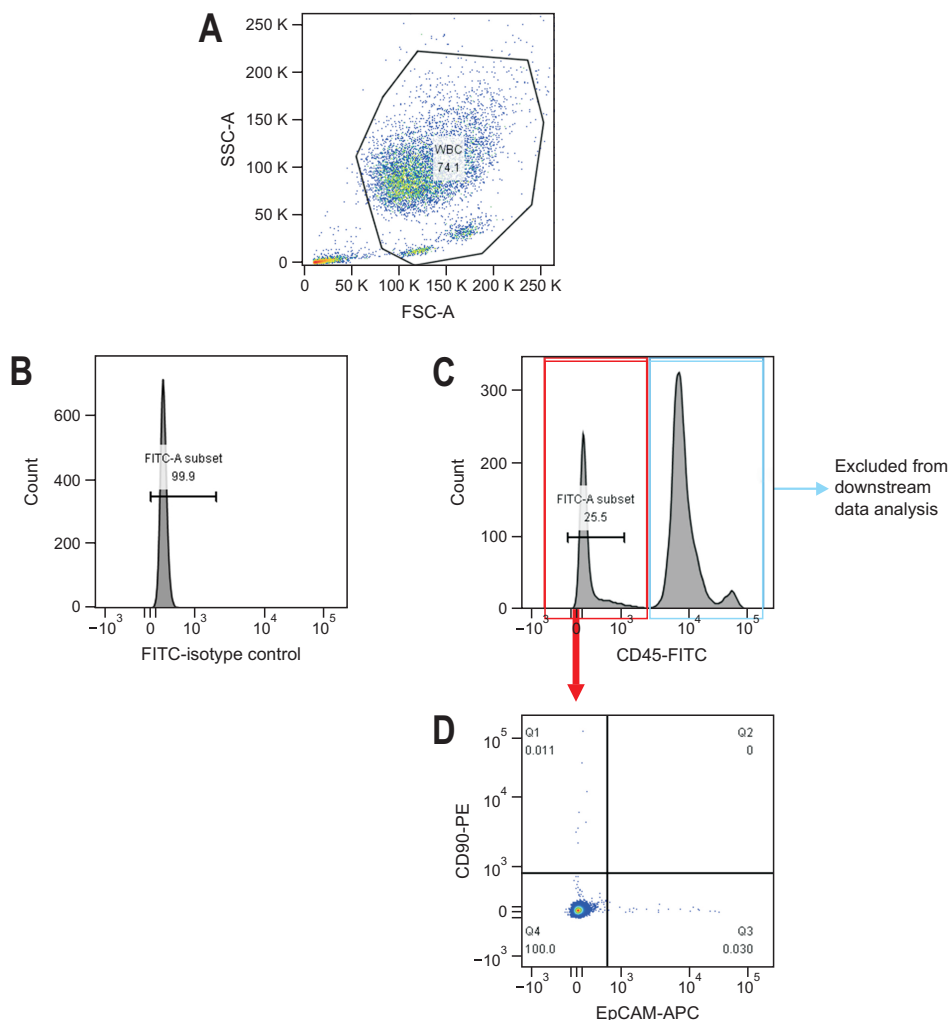
Fig. 1. Example of flow cytometry data analysis. The strategy followed to exclude hematopoietic cells with anti-CD45-FITC antibody is shown. (A) Forward scatter versus side scatter (FSC-A vs SSC-A) plot for total counted events; the gate excludes cell doublets and very large cells. (B) Histogram of isotype control using cells within the defined gate (A). (C) Cells within the defined gate (A) are represented in a histogram to evaluate the relative expression of CD45-FITC. (D) Cell counting was based on the expression of either EpCAM or CD90 in the triple-stained samples using CD45 negative cell population (red box in C).

WBC, white blood cell; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; EpCAM, epithelial cell adhesion molecule.

levels of stemness- and epithelial-mesenchymal transition (EMT)-related genes. The primary endpoint of this study was determining the correlation between disease-free survival (DFS) and isolated perioperative CTCs with cancer stemness from peripheral blood using FACs. DFS was calculated using the Kaplan-Meier method and the Cox proportional hazards model, and differences were compared using the log-rank test. All statistical analyses were performed using the statistical software SPSS version 23.0 for Windows (IBM Corp., Armonk, NY, USA), with $p<0.05$ considered statistically significant. Receiver operating characteristic curve was employed to identify optimal cutoff levels for CTC counts using the MedCalc program version 12.7.0 (MedCalc Software, Ostend, Belgium).

RESULTS

1. Clinicopathological characteristics of HCCs according to the expression status of EpCAM and/or CD90

Peripheral blood samples were collected from 25 patients with HCC who underwent LDLT before surgery and at postoperative days 1 and 7, and then, EpCAM+, CD90+, and EpCAM+/CD90+ CTCs were detected via FACs. In the blood samples before surgery, the average number of EpCAM+, CD90+, and EpCAM+/CD90+ CTCs were 10.25 (range, 1 to 37), 4.40 (range, 1 to 14) and 4.83 (range, 1 to 14), respectively. On postoperative day 1, the average number of EpCAM+, CD90+, and EpCAM+/CD90+ CTCs were 12.60 (range, 1 to 71), 4.60 (range, 1 to 18) and 1.83 (range, 1 to 4), respectively. On postoperative day 7 the average number of EpCAM+, CD90+, and EpCAM+/CD90+ CTCs were 4.69 (range, 1 to 15), 2.78 (range, 1 to 8), and 2.00 (range, 1 to 4), respectively. EpCAM, CD90 and co-expressed CTCs (≥1 per 10 mL blood) before surgery were detected in 12 (48%), 10 (40%), and six (24%)
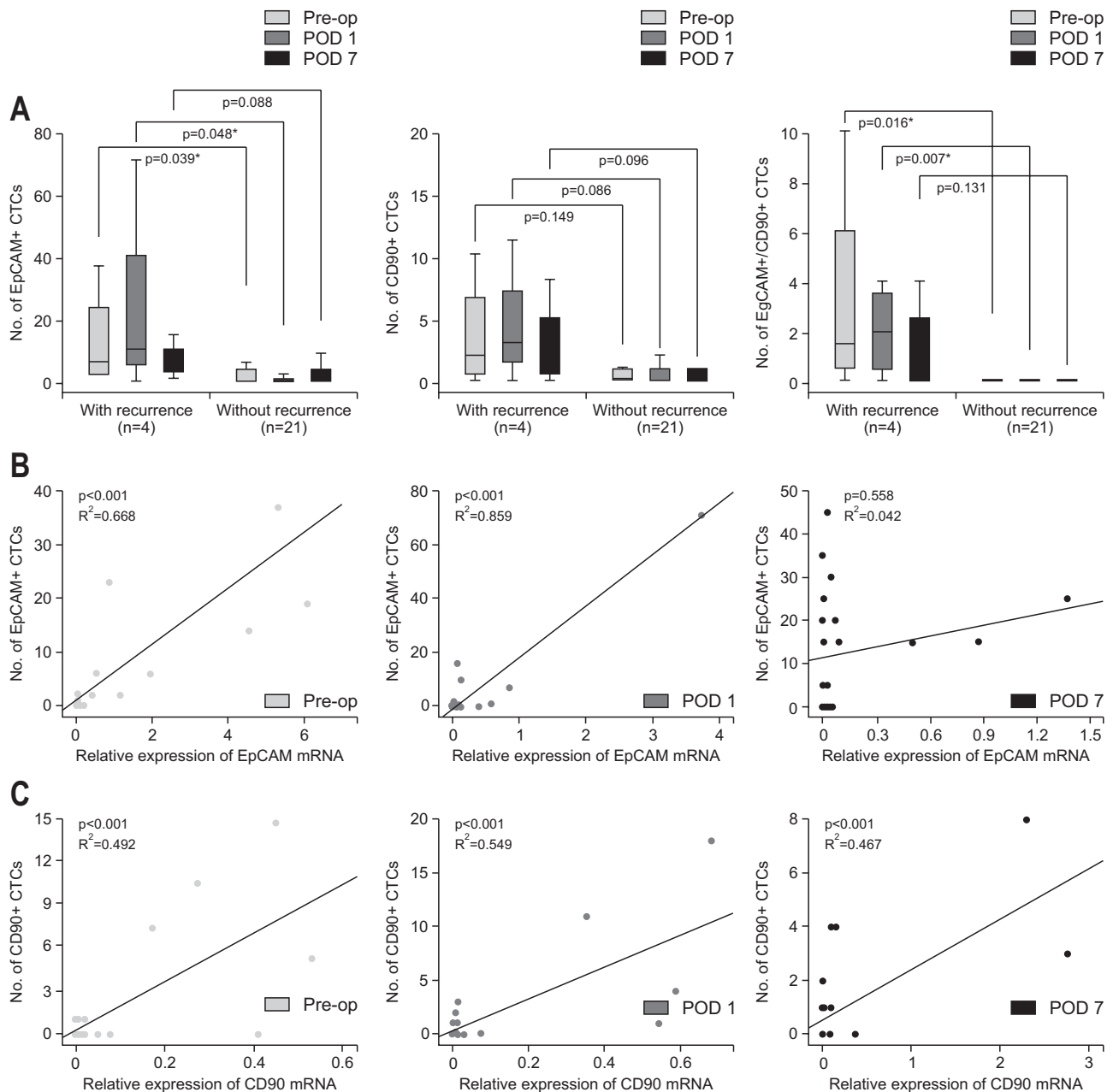
Fig. 2. Comparison of the number of circulating tumor cells (CTCs) with and without recurrence. Comparison of the number of CTCs expressing either EpCAM or CD90 and coexpressing EpCAM and CD90 in hepatocellular carcinoma patients with and without recurrence, on postoperative days 1 and 7 (A). The correlation between the number of CTCs expressing EpCAM and *EpCAM* mRNA levels is shown preoperatively and on postoperative days 1 and 7 (B). Dot blot showing the correlation with the number of CTCs expressing CD90 and mRNA levels of *CD90* preoperatively and on postoperative days 1 and 7 (C).
EpCAM, epithelial cell adhesion molecule; CD, cluster of differentiation; Pre-op, preoperative; POD, postoperative day; mRNA, messenger RNA.

patients, respectively. On postoperative day 1, EpCAM+, CD90+, and EpCAM+/CD90+ CTCs were detected in 11 (44%), 11 (44%), and six (24%) patients, respectively. On postoperative day 7, EpCAM+ or CD90+ CTCs were detected in 16 (64%) and nine (36%) patients, and EpCAM+/CD90+ CTCs were identified in five (20%) patients (Supplementary Table 1). Through plotting a receiver operating characteristic curve, the optimal cutoff level of

CTC counts, which represents the maximum sum of the sensitivity and specificity for predicting HCC recurrence, was found to be 1 (Supplementary Fig. 1).

The number of EpCAM+ CTCs in HCC patients with recurrence was significantly higher than in HCC patients without recurrence preoperatively (mean, 12.75 vs 3.79; p=0.039) and on postoperative day 1 (22.75 vs 1.90; p=0.048), whereas this difference was not significant by

postoperative day 7 (6.50 vs 2.53; p=0.088) (Fig. 2A left). The counts of CD90+ CTCs showed no significant difference between HCC patients with recurrence and those without preoperative, on postoperative days 1 and 7 (all p>0.05) (Fig. 2A middle). The number of EpCAM+/CD90+ CTCs in HCC with recurrence was significantly greater than that in HCC without recurrence preoperatively (3.25 vs 0.84; p=0.016) and on postoperative day 1 (2.00 vs 0.16; p=0.007), but not on postoperative day 7 (1.25 vs 0.28; p=0.131) (Fig. 2A right).

Potential correlations involving the detected numbers of EpCAM+ CTCs and levels of *EpCAM* mRNA in peripheral blood were also investigated. We found that the number of detected EpCAM+ CTCs was well matched with the mRNA levels of *EpCAM* in CD45-depleted peripheral blood mononuclear cells (R^2=0.668, p<0.001) on postoperative day 1 (R^2=0.859, p<0.001), but not on postoperative day 7 (R^2=0.042, p=0.558) (Fig. 2B). The mRNA levels of *CD90* in CD45-depleted peripheral blood mononuclear cells was strongly correlated with the number of CD90+ CTCs preoperatively (R^2=0.467, p<0.001), on postoperative day 1 (R^2=0.549, p<0.001), and on postoperative day 7 (R^2=0.492, p<0.001) (Fig. 2C).

Significant correlation of EpCAM+/CD90+ CTCs with MVI compared to cells expressing either EpCAM or CD90 alone or not preoperative, on postoperative days 1 and 7 (50.0%, 66.7%, and 60.0% were positive for MVI, with p=0.087, p=0.005, and p=0.035, respectively), and higher
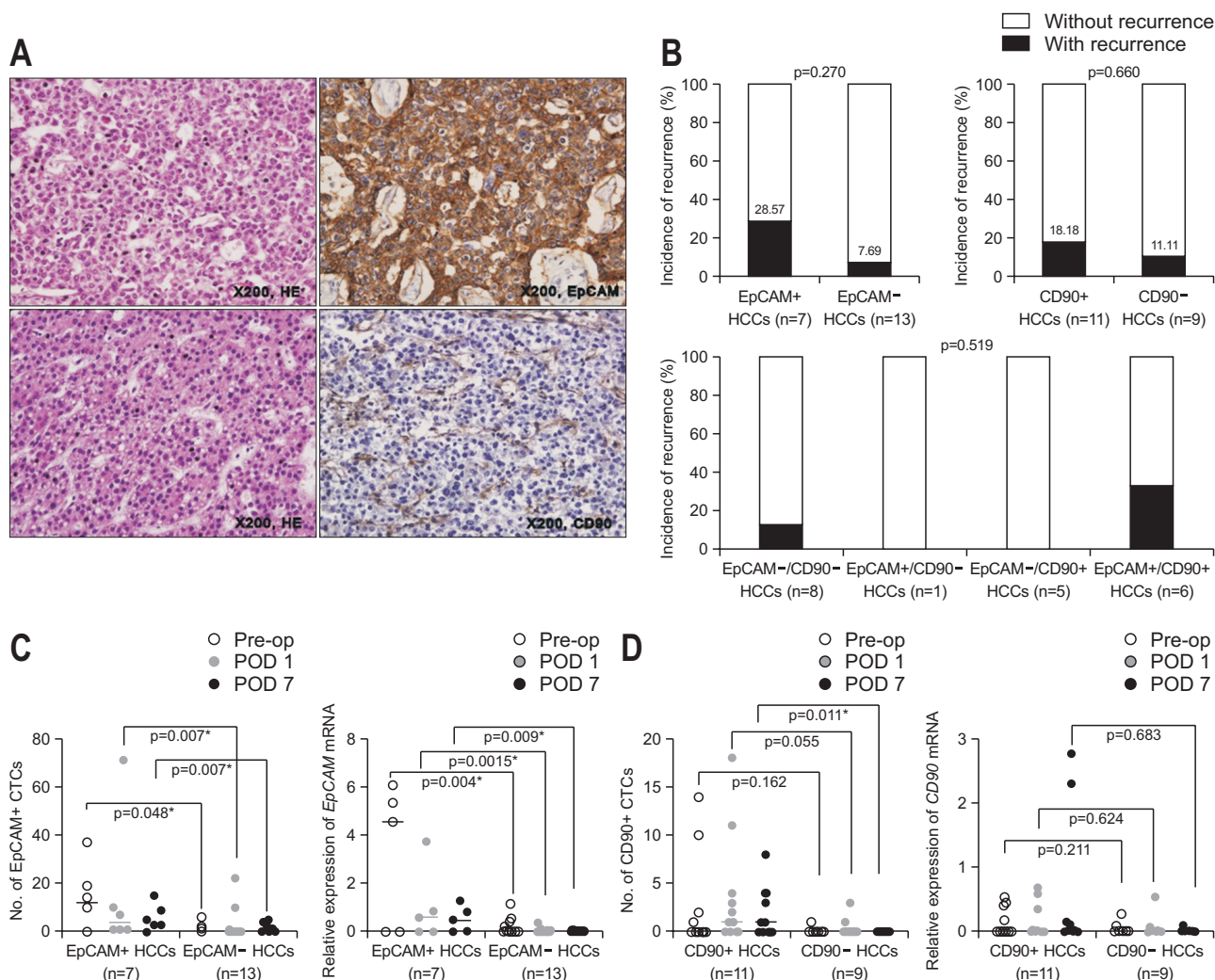

Fig. 3. Comparison of EpCAM-positive circulating tumor cells (CTCs) and mRNA levels of *EpCAM* according to the status of EpCAM expression in HCC tissues. (A) Representative images of EpCAM and CD90 expression (right, ×200) and H&E (left, ×200) in HCC. (B) Bar chart showing the incidence of recurrence according to EpCAM, CD90, EpCAM/CD90 detection in HCC tissue. Differences in EpCAM-expressing CTC numbers (C) and CD90-expressing CTC numbers (D) according to the respective EpCAM or CD90 protein expression status in HCC tissues assessed preoperatively and on postoperative days 1 and 7.

EpCAM, epithelial cell adhesion molecule; mRNA, messenger RNA; HCC, hepatocellular carcinoma; CD90, cluster of differentiation 90; Pre-op, preoperative; POD, postoperative day. *p<0.05.

correlation of Child-Pugh class with EpCAM+ CTCs on postoperative day 1 (p=0.023) were observed, but not with other clinical characteristics, such as age, gender, etiology, Model for End-Stage Liver Disease score, preoperative serum AFP >400 ng/mL, preoperative serum protein induced by vitamin K absence-II (PIVKA-II) >100 mAU/mL, Milan criteria, Barcelona Clinic Liver Cancer stage at LT, United Network for Organ Sharing stage at LT, pretransplant treatment, or pathologic Edmondson-Steiner grade (all p>0.05) (Supplementary Table 2).

2. Comparison of EpCAM+/CD90+ CTCs and *EpCAM/CD90* mRNA levels in peripheral blood according to EpCAM/CD90 protein expression status in HCCs

Immunohistochemical staining for EpCAM and CD90 was performed in surgically removed human HCC tissues (Fig. 3). The recurrence was compared to the status of EpCAM and/or CD90 expression of HCCs. EpCAM protein was expressed in seven of 20 (35.0%) HCCs (Fig. 3B). Two of EpCAM expressed HCCs were shown pantumoral

expression, while two patients were peritumoral EpCAM expression. The remaining three cases were shown focal expression of EpCAM without specific pattern in the intratumoral region (Supplementary Fig. 2). Two out of seven EpCAM+ HCCs recurred (28.57%), and one recurred in 13 EpCAM- HCCs (7.69%) (Fig. 3B). However, the pattern of EpCAM expression was not associated with the recurrence. Moreover, the number of EpCAM+ CTCs counted by FACs was statistically higher in EpCAM+ HCCs than in those without expression of EpCAM protein, and this statistical difference was identified in preoperative and postoperative day 1 samples, and postoperative day 7 samples (all p<0.05) (Fig. 3C). *EpCAM* transcripts were higher in EpCAM+ HCCs than in EpCAM– HCCs (all p<0.05) (Fig. 3C).

The expression of CD90 was detected in the stellate cells of HCCs and eleven (55%) out of 20 HCCs showed CD90 expression (Fig. 3). There was no significant difference of CD90 expression between HCCs with recurrence and those without recurrence. The number of CD90+ CTCs counted
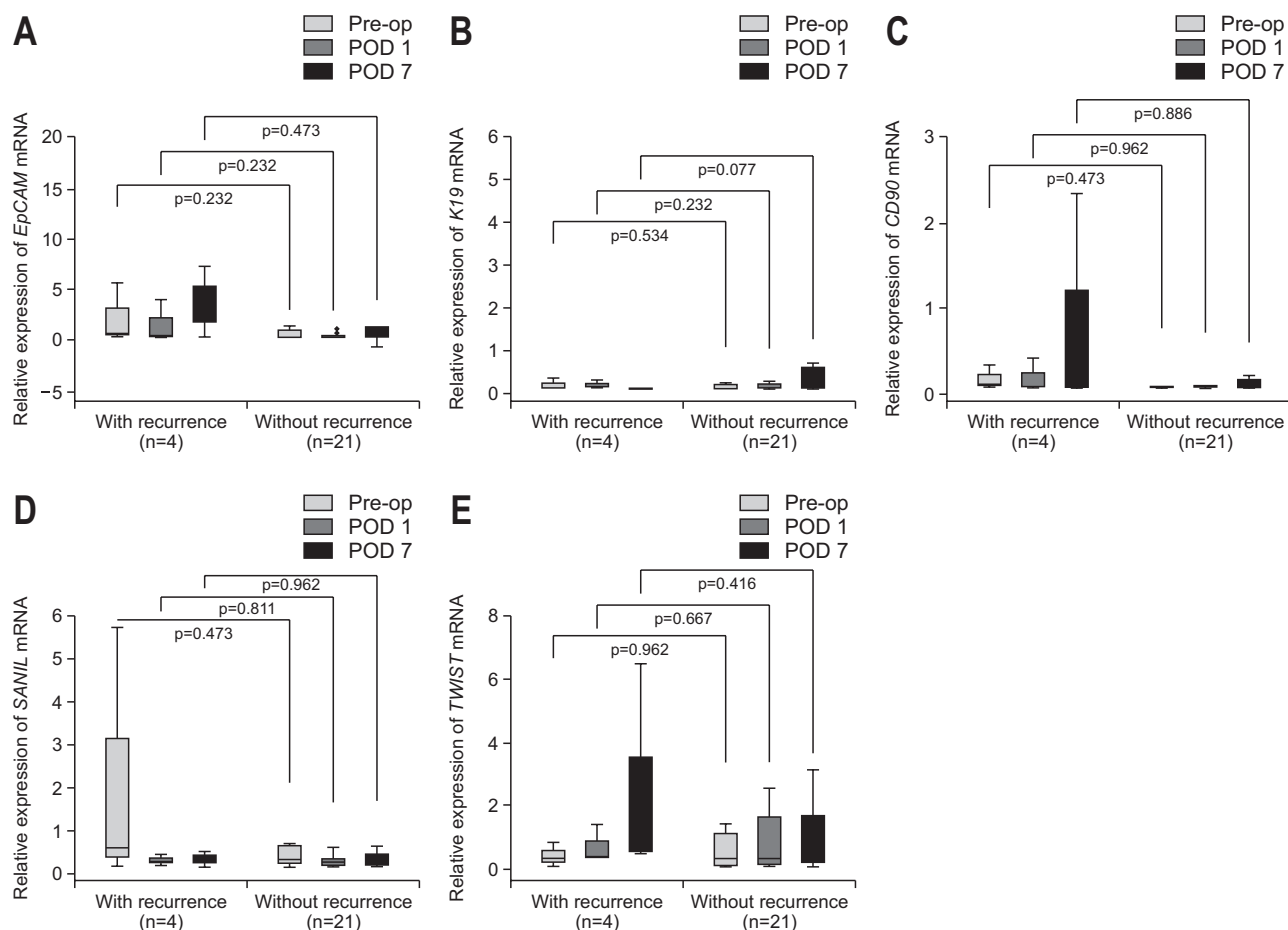

Fig. 4. Differences in the mRNA levels of stemness- and EMT-related genes in patients with and without recurrence. Box plot showing the mRNA levels of (A) *EpCAM*, (B) *K19*, (C) *CD90*, (D) *SNAIL*, and (E) *TWIST* preoperatively and at postoperative days 1 and 7.
mRNA, messenger RNA; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; K19, keratin 19; CD90, cluster of differentiation 90; Pre-op, preoperative; POD, postoperative day.

Table 2. Univariate Analysis of Factors Predictive of Recurrence or Disease-Free Survival

Characteristics	No. of patients (recurrence)	Disease-free survival, %			p-value
		1 Year	3 Years	5 Years	
Age					0.620
≤50 yr	4 (1)	75.0	75.0	75.0	
>50 yr	21 (3)	90.5	85.7	85.7	
Sex					0.541
Male	23 (4)	87.0	82.6	82.6	
Female	2 (0)	100.0	100.0	100.0	
Etiology					0.396
HBV	22 (3)	90.9	86.4	86.4	
HCV	1 (0)	100.0	100.0	100.0	
NBNC	2 (1)	50.0	50.0	50.0	
Cirrhosis					0.298
Yes	20 (4)	85.0	80.0	80.0	
No	5 (0)	100.0	100.0	100.0	
Pretransplant liver operation					0.094
Yes	5 (2)	60.0	60.0	60.0	
No	20 (2)	95.0	90.0	90.0	
Milan criteria					0.289
Within	18 (2)	88.9	88.9	88.9	
Beyond	7 (2)	85.7	71.4	71.4	
Pretransplant serum AFP					0.673
≥400 ng/mL	1 (0)	100.0	100.0	100.0	
<400 ng/mL	24 (4)	87.5	83.3	83.3	
Pretransplant serum PIVKA-II					0.047*
≥100 mAU/mL	8 (3)	75.0	62.5	62.5	
<100 mAU/mL	17 (1)	94.1	94.1	94.1	
Preoperative EpCAM+					0.025*
Yes	12 (4)	75.0	66.7	66.7	
No	13 (0)	100.0	100.0	100.0	
POD 1 EpCAM+					0.156
Yes	11 (3)	72.7	72.7	72.7	
No	14 (1)	92.9	92.9	92.9	
POD 7 EpCAM+					0.114
Yes	16 (4)	81.3	75.0	75.0	
No	9 (0)	100.0	100.0	100.0	
Preoperative EpCAM+/CD90+					0.004*
Yes	6 (3)	50.0	50.0	50.0	
No	19 (1)	94.7	94.7	94.7	
POD 1 EpCAM+/CD90+					0.004*
Yes	6 (3)	50.0	50.0	50.0	
No	19 (1)	100.0	94.7	94.7	
POD 7 EpCAM+/CD90+					0.077
Yes	5 (2)	60.0	60.0	60.0	
No	20 (2)	95.0	90.0	90.0	
Preoperative CD90+					0.104
Yes	10 (3)	70.0	70.0	70.0	
No	15 (1)	100.0	93.3	93.3	
POD 1 CD90+					0.156
Yes	11 (3)	72.7	72.7	72.7	
No	14 (1)	100.0	92.9	92.9	
POD 7 CD90+					0.083
Yes	9 (3)	77.8	66.7	66.7	
No	16 (1)	93.8	93.8	93.8	
MVI					0.519
Yes	6 (2)	66.7	66.7	66.7	
No	19 (2)	93.3	86.7	86.7	

Table 2. Continued

Characteristics	No. of patients (recurrence)	Disease-free survival, %			p-value
		1 Year	3 Years	5 Years	
Tumor size					0.498
≤5 cm	19 (4)	84.2	78.9	78.9	
>5 cm	2 (0)	100.0	100.0	100.0	
Tumor number					0.262
Single	6 (2)	83.3	66.7	66.7	
Multiple	17 (2)	88.2	88.2	88.2	
Edmondson–Steiner grade					0.713
I-II	12 (2)	91.7	83.3	83.3	
III-IV	9 (2)	77.8	77.8	77.8	
NA	4 (0)				
Post-transplant usage of mTORi					0.466
Yes	16 (2)	93.8	87.5	87.5	
No	9 (2)	77.8	77.8	77.8	

HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B, non-C hepatitis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence-II; EpCAM, epithelial cell adhesion molecule; POD, postoperative day; CD90, cluster of differentiation 90; MVI, microvascular invasion; NA, not available; mTORi, mammalian target of rapamycin inhibitor.
*Statistically significant, p<0.05.

on postoperative day 7 samples was higher in HCCs with CD90 expression than in those without (p=0.011). This difference was not identified preoperative and on postoperative day 1 samples (all p<0.05). The mRNA levels of *CD90* showed no significant difference between CD90+ HCCs and CD90– HCCs preoperative, on postoperative days 1 and 7 samples (Fig. 3D). Furthermore, CD90 expression was more frequent in EpCAM+ HCCs compared to EpCAM– HCCs. The status of EpCAM and/or CD90 expression were classified as (1) EpCAM+ and CD90+, (2) EpCAM+ and CD90–, (3) EpCAM– and CD90+, or (4) EpCAM– and CD90–. The recurrence rate was not shown any differences among these four groups (Fig. 3B).

3. mRNA levels of stemness- and EMT-related genes in HCC recurrence after LDLT

The expression levels of stemness-related genes (*EpCAM, K19,* and *CD90)* and EMT-related genes (*SNAIL* and *TWIST*) were assessed via RT-qPCR in CD45-depleted peripheral blood mononuclear cells of 25 patients preoperatively, on postoperative day 1, and postoperative day 7 (Fig. 4).

The transcripts of *EpCAM* and *CD90* were relatively higher in HCC with recurrence than in HCC without recurrence; however, statistical significance was not reached (preoperative, postoperative day 1, and postoperative day 3, all p>0.05) (Fig. 4). However, *K19* mRNA levels did not exhibit any difference between the two groups. Similar to *K19*, the transcripts of *SNAIL* and *TWIST* did not show any differences between HCCs with and without recurrence.

4. Prognostic factors for DFS in HCC after LDLT

Univariate analysis showed that the level of pretransplant serum PIVKA-II ≥100 mAU/mL was a significant variable affecting DFS (p=0.047), and EpCAM+ CTCs were associated with decreased DFS preoperatively (p=0.025) (Table 2, Fig. 5), but not on postoperative days 1 and 7. EpCAM+/CD90+ CTCs were associated with significantly shorter DFS preoperatively and at postoperative day 1 (both p=0.004) (Table 2, Fig. 5). DFS was not significantly different according to the CD90 expression status of CTCs at any time point. For LT patients, any clinicopathological characteristics such as high pretransplant serum levels of AFP, Milan criteria, tumor size, number of tumors, Edmondson–Steiner grade, and post-transplantation usage of mTORi were not associated with DFS and overall survival (Fig. 5, Table 2).

Multivariate analysis using the Cox proportional hazards model revealed that preoperative PIVKA-II and postoperative day 1 EpCAM+/CD90+ CTCs act as independent risk factors for HCC recurrence (hazard ratio, 14.64; 95% confidence interval, 1.08 to 198.20; p=0.043 and hazard ratio, 26.88; 95% confidence interval, 1.86 to 387.51; p=0.016, respectively) (Table 3). The sensitivity, specificity, accuracy, positive predictive value, and negative predictive value for postoperative day 1 EpCAM+/CD90+ CTCs and pretransplant PIVKA-II levels for predicting HCC recurrence are shown in Table 4. Postoperative day 1 EpCAM+/CD90+ CTCs≥1 has high specificity of 94.7%.
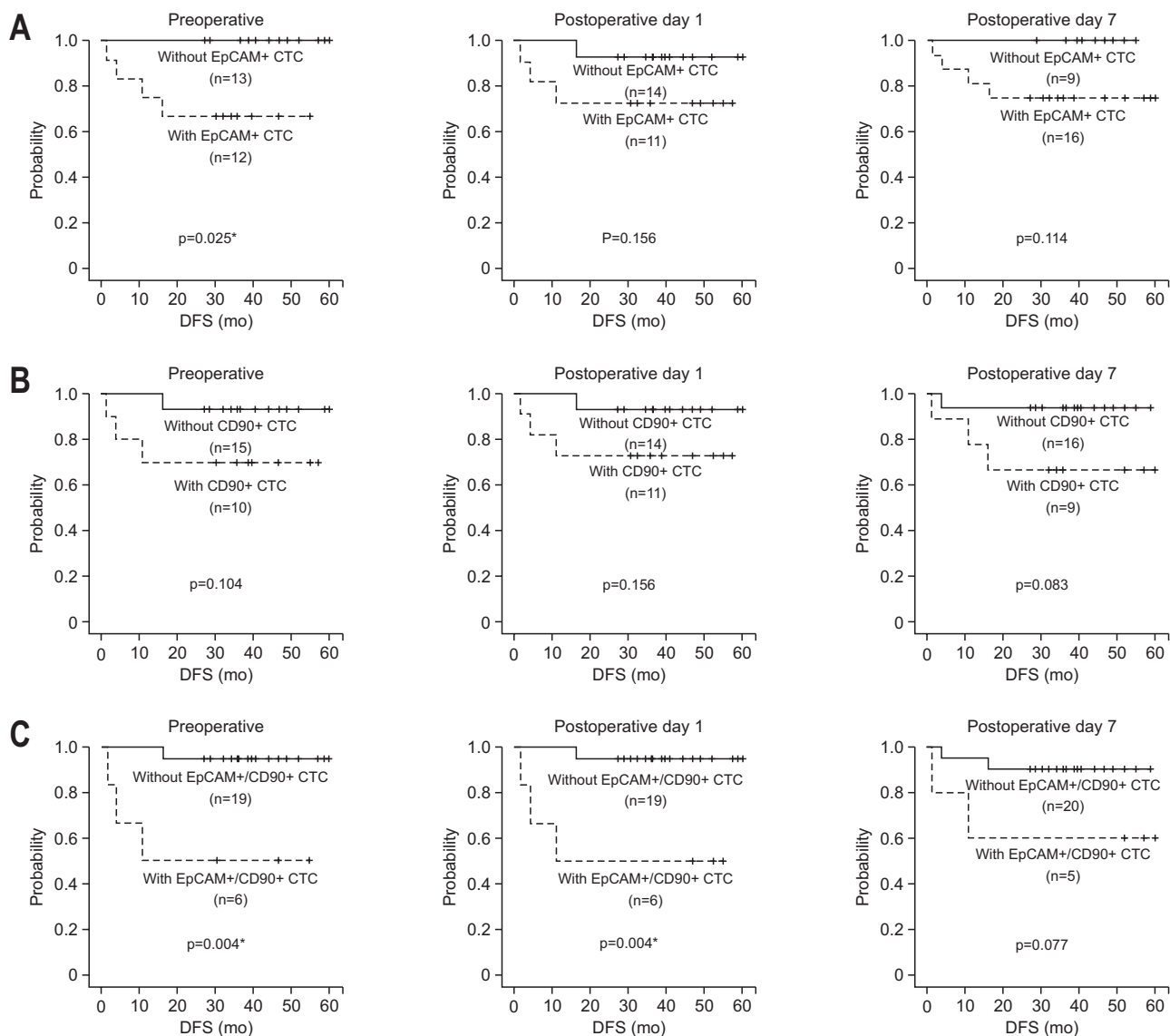
Fig. 5. Survival analysis plots for DFS in HCC according to the expression status of EpCAM and/or CD90 in CTCs. (A) Survival analysis plots for DFS in HCC according to the expression status of EpCAM in CTCs preoperatively and at postoperative days 1 and 7. (B) Survival analysis plots for DFS in HCC according to the expression status of CD90 in CTCs preoperatively and on postoperative days 1 and 7. (C) Survival analysis plots for DFS in HCC according to the coexpression status of EpCAM and CD90 in CTCs preoperatively and on postoperative days 1 and 7.
DFS, disease-free survival; HCC, hepatocellular carcinoma; EpCAM, epithelial cell adhesion molecule; CD90, cluster of differentiation 90; CTCs, circulating tumor cells. *p<0.05.

Table 3. Multivariate Analysis for Hepatocellular Carcinoma Recurrence after Living Donor Liver Transplantation

Characteristics	Hazard ratio (95% CI)	p-value
Pretransplant serum PIVKA-II ≥100 mAU/mL	14.64 (1.08–198.20)	0.043
POD 1 EpCAM and CD90 positive	26.88 (1.86–387.51)	0.016

CI, confidence interval; PIVKA-II, protein induced by vitamin K absence-II; POD, postoperative day; EpCAM, epithelial cell adhesion molecule; CD90, cluster of differentiation 90.

DISCUSSION

Several markers in HCC have been demonstrated to present stem cell-like properties in previous studies.[17]

Among such markers, EpCAM and CD90 were evaluated to isolate CTCs with cancer stemness, and the detection of HCC with these markers was strongly associated with HCC recurrence after surgical resection.[9,10] With the ex-

Table 4. Diagnostic Performance of CTCs and PIVKA-II Levels in the Prediction of Hepatocellular Carcinoma Recurrence

Characteristics	Sensitivity	Specificity	Accuracy	PPV	NPV
POD 1 EpCAM+/CD90+ CTCs ≥1	50.0 (3/6)	94.7 (18/19)	84.0 (21/25)	75.0 (3/4)	85.7 (18/21)
Pretransplant serum PIVKA-II ≥100 mAU/mL	37.5 (3/8)	94.1 (16/17)	76.0 (19/25)	75.0 (3/4)	76.2 (16/21)

Data are presented as percentages (number/number).
CTC, circulating tumor cell; PIVKA-II, protein induced by vitamin K absence-II; POD, postoperative day; EpCAM, epithelial cell adhesion molecule; CD90, cluster of differentiation 90; PPV, positive predictive value; NPV, negative predictive value.

tension of these two studies and our previous research,[11] we investigated whether the detection of CTCs with cancer stemness using FACs with EpCAM and CD90 markers could predict HCC recurrence after LDLT.

EpCAM, an epithelial cell adhesion molecule, is a well-established cancer stem cell in many other cancers as well as HCC.[10,18] CD90 is also a cancer stem cell marker in HCC; however, it has features of vascular endothelial cells and is associated with a mesenchymal metastatic cancer stem cell marker.[9] Yamashita *et al.*[16] demonstrated that EpCAM+ and CD90+ HCC has a specific gene expression pattern, and has stem cell features with high capacity for metastasis to the lung in a xenotransplantation model. In this study, the detection of EpCAM+/CD90+ cells in peripheral blood on postoperative day 1 was associated with a higher rate of MVI in the primary HCC. The detected numbers of EpCAM+ CTCs and EpCAM+/CD90+ CTCs were significantly higher in patients with HCC recurrence than in those without recurrence preoperatively and on postoperative day 1. In agreement with previous studies, the detection of both EpCAM+ and CD90+ CTCs compared to EpCAM+ alone preoperatively and at postoperative day 1 was found to be more associated with HCC recurrence after LDLT in our study. The detected number of EpCAM+ and CD90+ CTCs showed a positive correlation with the mRNA levels of these markers in peripheral blood. However, the mRNA levels of *EpCAM, K19*, and *CD90* and EMT-related genes (*SNAIL* and *TWIST*) were not associated with HCC recurrence after LDLT.

Potential correlations involving EpCAM protein expression in HCC tissues, the detected numbers of EpCAM+ CTCs, and the levels of *EpCAM* mRNA in peripheral blood were also investigated. In our study, HCCs with EpCAM+ protein expression were found to have significantly higher numbers of EpCAM+ CTCs and higher EpCAM mRNA levels during the perioperative period. These results indicated that EpCAM+ CTCs mainly originated from EpCAM+ HCC tumors. However, the number of EpCAM+ CTCs and EpCAM+/CD90+ CTCs on postoperative day 7 showed less correlation with HCC recurrence compared to those seen preoperatively and at postoperative day 1. The detection of EpCAM+ CTCs and EpCAM+/CD90+ CTCs was also associated with HCC recurrence only preopera-

tively and on postoperative day 1. These results might be attributed to the fact that these HCC cancer stem cell markers are also those of normal hepatocyte progenitor cells, which play a major role in liver regeneration, and that the grafted liver is actively regenerated on postoperative day 7.[19-22]

The prognostic impact of EpCAM+/CD90+ CTC detection on the preoperative day in this study can be incorporated into clinical practice because liquid biopsy is an emerging method to analyze tumor biology in a minimally invasive manner, and has a potential impact on LT candidate selection and postoperative monitoring.[12,14] The selection criteria for LT candidates among HCC patients have evolved from classic morphological to biological criteria, which include tumor markers and molecular biomarkers. However, most molecular biomarkers are investigated and established in tumor tissues, and tissue biopsy is required for preoperative clinical use. FACs for the detection of CTCs is a reliable high-throughput technology with objectivity and high efficiency for detecting rare cell population in fluids.[23] However, this method requires a large number of cells for accurate quantitation and there is no capacity for morphological analysis without additional instruments such as fluorescent microscope.[24-26] Furthermore, it has been also suggested that FACs may have limited sensitivity for CTC analysis in human blood samples if one or two cells were contained in the samples.[27] To overcome this limitation, we increased the amount of blood used for FACs analysis to 10 mL, and proceeded with RT-qPCR as well as FACs. The number of EpCAM+ CTCs were positively correlated with mRNA level of EpCAM and that of CD90+ CTCs was shown positive correlation with mRNA level of CD90. Isolation of CTCs represents one of the efforts to identify the direct cause of HCC tumor recurrence, and can be more useful for selecting optimal candidates for LT in HCC patients who fall outside of accepted clinical criteria, or in patients with down-staging treatments for advanced HCC. In multivariate analysis, the detection of EpCAM+/CD90+ CTCs at postoperative day 1 and pretransplant serum PIVKA-II ≥100 mAU/mL were found to be independent prognostic factors for HCC recurrence after LDLT in this study, which can be used to select high-risk LT candidates and optimal post-transplant immunosuppressive drugs. A recent meta-analysis demonstrated

that postoperative immunosuppression based on mTORi administration improved DFS and overall survival compared with that based on CNI.[28] mTORi without CNI may be clinically recommended in these patients with higher risk of HCC recurrence after LT; however, further well-designed studies are required for additional solid evidence.

This study had several limitations. Even though this study was designed in a prospective way, the number of study subjects and patients with HCC recurrence was small, which might produce the question about the reliability of the results by statistical analysis using the Cox proportional hazard model. In addition, conventional well-known prognostic factors such as tumor size, number, MVI, and tumor histologic grade were not associated with HCC recurrence after LDLT in this study, which may mean that this study cohort might not represent all transplant recipients, and these results might not be generalizable in clinical practice. However, despite the small study population and events, the detection of EpCAM+/CD90+ CTCs before surgery and at postoperative day 1 aided in predicting HCC recurrence after LDLT in this study. This result is in agreement with the findings of previous studies showing that circulating cancer stem cells are directly responsible for metastasis and recurrence, and are more powerful in predicting tumor recurrence compared with conventional clinicopathological prognostic factors.[2,3,14,29-33] Therefore, the preliminary results of this study should be validated in a large-scale multicenter study.

In conclusion, pretransplant serum PIVKA-II levels and the detection of EpCAM+ CTCs preoperatively and EpCAM+/CD90+ CTCs preoperatively and on postoperative day 1 could predict HCC recurrence after LDLT. These findings can be used to select optimal candidates for LT in patients with HCC, especially those at higher risk of recurrence, and can guide tailored postoperative monitoring and immunosuppression. However, due to the small size of the study cohort and the events, these preliminary results should be validated in a large prospective study.

CONFLICTS OF INTEREST

Y.N.P. is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

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AUTHOR CONTRIBUTIONS

Conceptualization: H.S.H., J.E.Y., G.H.C., Y.N.P. Data curation: H.S.H., J.E.Y., G.H.C. Formal analysis: H.S.H., J.E.Y., G.H.C. Methodology: J.E.Y., G.H.C., Y.N.P. Visualization: H.S.H., J.E.Y. Sample collection: D.H.H., J.S.C., J.G.L., D.J.J., M.S.K., S.I.K., G.H.C. Funding acquisition: G.H.C., Y.N.P. Writing - original draft: H.S.H., J.E.Y. Writing - review & editing: G.H.C., Y.N.P. Approval of final manuscript: all authors.

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SUPPLEMENTARY MATERIALS

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