Analysis of circulating tumor cells in patients with hepatocellular carcinoma recurrence following liver transplantation

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ABSTRACT

Although studies have shown that detection of peripheral circulating tumor cells (CTCs) is an important tool for monitoring prognosis and therapeutic response in patients with cancer, few studies have analyzed their role in patients with hepatocellular carcinoma (HCC) following liver transplantation (LTx). The present study examined whether CTC levels were associated with HCC recurrence in patients with HCC after LTx. This prospective study included 47 patients who received LTx between October 2014 and May 2016 and who underwent analysis for peripheral CTCs at least twice using the CanPatrol system. Baseline Edmondson stage, T stage, accumulated tumor diameter, microvascular cancer embolus, and alpha-fetoprotein (AFP) levels were greater in patients with recurrence (all $p<0.05$). In addition, 70.2% of patients with HCC were CTC-positive. Although the proportion of CTC subtypes changes following LTx and over the follow-up period with increased epithelial and interstitial CTC levels, no significant associations were observed between change in total CTCs or CTC subtype and HCC recurrence (all $p>0.05$). In conclusion, baseline Edmondson stage, T stage, accumulated tumor diameter, microvascular cancer embolus, and AFP levels may be predictive of HCC recurrence following LTx; however, CTC levels and subtypes were not. Further large, multicenter studies are necessary to confirm these results.

Significance of this study

What is already known about this subject?

- Circulating tumor cells (CTCs) are closely related to the metastasis and recurrence of various cancers.
- CTCs are used to evaluate tumor invasiveness and therapeutic efficacy.
- The prognostic value of blood CTCs in patients with hepatocellular carcinoma (HCC) has been demonstrated.
- The number of CTCs is related to the overall survival of patients with HCC.

What are the new findings?

- 70.2% of patients with HCC were CTC-positive.
- The proportion of CTC subtypes changes following liver transplantation (LTx).
- Epithelial and interstitial CTC levels elevated over the follow-up period.

How might these results change the focus of research or clinical practice?

- The current study indicated that CTC levels and subtypes were not predictive of HCC recurrence following LTx.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignancy with high morbidity and mortality. In China, HCC is the third leading cause of cancer-related death, following gastric cancer and esophageal cancer.[1 2] Liver transplantation (LTx) and hepatectomy are widely accepted as effective strategies to cure HCC.[3] LTx involves resection of as much of the tumor as possible, and cures hepatic cirrhosis, which is also detected in >80% of patients with HCC. Notably, the 5-year survival rate of patients with HCC following LTx ranged from 46.6% to 60.9%.[4–6]

Hematogenous metastasis is the major route of extrahepatic HCC metastasis, especially via the portal venous system. Even in the early stage of primary HCC, cancer cells may shed and enter the circulation, leading to the distal spread, implantation, and formation of metastatic foci.[7] Postoperative metastasis and recurrence are important factors, affecting the therapeutic efficacy of LTx in patients with HCC[8 9] as well as the prognosis of patients with HCC. Therefore, a method for monitoring metastasis and therapeutic efficacy in patients with HCC is of high clinical significance.

In recent years, increasing attention has been paid to the role of circulating tumor cells (CTCs) in the metastasis of malignancies.[10 11] Studies have shown that CTCs are closely related to the metastasis and recurrence of breast cancer, colorectal cancer, and non-small cell lung cancer.[12–14] Moreover, CTCs have become an important tool in the clinical treatment of cancers,[15] as well as a tool for evaluating tumor invasiveness and therapeutic efficacy.[16–18] Thus, accurate detection of peripheral CTCs may be employed as a prognostic tool to evaluate therapeutic response, guiding therapeutic protocol selection.[19] For the first time,

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Vona *et al*[20] used the isolation by size of epithelial tumor cells method to investigate the prognostic value of blood CTCs in 44 patients with HCC, which showed that the presence of peripheral CTCs was closely associated with prognosis and disease recurrence in patients with HCC. With improvements in CTC separation methods, more studies have revealed that preoperative or postoperative detection of CTCs is closely related with HCC recurrence and metastasis, and the number of CTCs is significantly different between patients with intrahepatic metastasis and those with extrahepatic metastasis.[21] The difference in the number of CTCs is also related to the progression-free survival (PFS) and overall survival (OS) of patients with HCC.[22]

In patients with HCC, CTCs can be divided into four types: epithelial, interstitial, mixed, and circulating tumor microemboli (CTM). Recent studies indicate that interstitial CTCs have more potent metastatic and invasive activities as compared with epithelial CTCs and are also resistant to apoptosis and aging.[23 24] Cells in CTM are largely non-proliferative and, therefore, possess more capability to resist the toxicity of cytotoxic drugs. As compared with single CTCs, CTMs are more likely to induce metastasis.[23 25 26] Thus, analysis of the prognostic value of CTC types may provide evidence on the clinical significance of CTC detection.

Because CTCs have not been analyzed in patients with HCC following LTx, the present study examined the number of peripheral CTC types before and after LTx and determined if CTC levels were associated with HCC recurrence after LTx. These data will explore whether the number and subtypes of CTCs can be used as a predictive factor for recurrence after LTx in patients with HCC. This approach may be used to evaluate the therapeutic efficacy of LTx in patients with HCC. In this scenario, once the high-risk patients are identified, the treatment intensity (such as chemotherapy) and the follow-up frequency may be increased.

MATERIALS AND METHODS

Study subjects

In this prospective study, a total of 47 patients who received LTx in the Department of Liver Transplantation Center of Guangzhou General Hospital of Guangzhou Military Region between October 2014 and May 2016 and who underwent analysis for peripheral CTCs at least twice were enrolled. Patients in group 1 (n=20) had their CTCs analyzed before transplantation and at 1 month after transplantation. Patients in group 2 (n=27) had their CTCs analyzed at 1 and 3 months after transplantation.

Patients were included if they met the following inclusion criteria: 18–70 years of age, a diagnosis of primary HCC confirmed by pathological examination, had no other tumors and no sign of liver cancer metastasis to other organs as confirmed by positron emission tomography (PET)-CT examination prior to transplantation, and with follow-up data following LTx. Patients with other tumors or having signs of liver cancer metastasis to other organs were excluded. All included patients were hepatitis B virus (HBV)-positive as confirmed by routine preoperative test; therefore, all included patients had HBV-induced HCC. Informed consent was collected from each subject.

Table 1 Sequences of the nucleic acid probes used in this study

Gene	Sequence (5'→3')
EpCAM	TGGTGCTCGTTGATGAGTCAAGCCAGCTTTGAGCAAATGA
	AAAGCCCATCATTGTTCTGGCTCTCATCGCAGTCAGGATC
	TCCTTGTCTGTTCTTCTGACCTCAGAGCAGGTTATTTCAG
CK8	CGTACCTTGTCTATGAAGGAACTTGGTCTCCAGCATCTTG
	CCTAAGGTTGTTGATGTAGCCTGAGGAAGTTGATCTCGTC
	CAGATGTGTCCGAGATCTGGTGACCTCAGCAATGATGCTG
CK18	AGAAAGGACAGGACTCAGGCGAGTGGTGAAGCTCATGCTG
	TCAGGTCCTCGATGATCTTGCAATCTGCAGAACGATGCGG
	AAGTCATCAGCAGCAAGACGCTGCAGTCGTGTGATATTGG
CK19	CTGTAGGAAGTCATGGCGAGAAGTCATCTGCAGCCAGACG
	CTGTTCCGTCTCAAACTTGGTTCTTCTTCAGGTAGGCCAG
	CTCAGCGTACTGATTTCCTCGTGAACCAGGCTTCAGCATC
Vimentin	GAGCGAGAGTGGCAGAGGACCTTTGTCGTTGGTTAGCTGG
	CATATTGCTGACGTACGTCAGAGCGCCCCTAAGTTTTTAA
	AAGATTGCAGGGTGTTTTCGGGCCAATAGTGTCTTGGTAG
Twist	ACAATGACATCTAGGTCTCCCTGGTAGAGGAAGTCGATGT
	CAACTGTTCAGACTTCTATCCCTCTTGAGAATGCATGCAT
	TTTCAGTGGGCTGATTGGCACTTACCATGGGTCCTCAATAA
CD45	TCGCAATTCTTATGCGACTCTGTCATGGAGACAGTCATGT
	GTATTTCCAGCTTCAACTTCCCATCAATATAGCTGGCATT

EpCAM, epithelial cell adhesion molecule.

Detection of CTCs

The CanPatrol (Yishan, Guangzhou, China) method was used to detect CTCs in the peripheral blood of patients with HCC. In brief, CTCs in 5 mL of blood samples were separated and enriched by filtration according to differences in cell size between CTCs and white blood cells as previously described.[27]

Following resuspension in posphate-buffered saline (PBS) and fixation with formaldehyde at room temperature for 60 min, multiple mRNA in situ analyses were employed to identify the specific nucleic acids in the enriched CTCs without relying on amplification of target sequences, resulting in the phenotyping and identification of CTCs.[27] Specifically, after permeabilization and digestion with a protease (Qiagen, Hilden, Germany), the samples were incubated for 2 hours at 42°C with capture probes specific for the epithelial biomarkers, epithelial cell adhesion molecule (EpCAM) and CK8/18/19, the mesenchymal biomarkers, vimentin and twist, and the leukocyte biomarker, CD45 (table 1).[27] After sequential incubation with a preamplifier solution, an amplifier solution, fluorescent-labeled probes, and an antiquenching solution (containing 4',6-diamidino-2-phenylindole (DAPI)), the samples were then observed under a microscope.

Red fluorescence is indicative of epithelial CTCs with the following marker profile: intracellular CD45-negative, EpCAM-positive, and CK8/18/19-positive. Green fluorescence is indicative of interstitial CTCs with the following marker profile: intracellular CD45-negative, vimentin-positive, and twist-positive. A combination of red and green fluorescence is indicative of mixed CTCs.[26] CTC positivity/negativity was defined according to the following criteria: CTC-positive, number of CTCs ≥2/5 mL; and CTC-negative: number of CTCs <2/5 mL.

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Follow-up analysis

Follow-up was performed in all 47 patients by telephone or in person during hospital visit. Liver function analysis, dynamic alpha-fetoprotein (AFP), routine blood tests, and abdominal color Doppler ultrasonography, CT, or MRI (PET-CT, if necessary) were performed for evaluation. According to clinical findings, postoperative recurrence was defined as follows: (1) AFP returned to normal level after surgery, but increased significantly, or AFP increased postoperatively in AFP-negative patients. Patients with active hepatitis, pregnancy, and reproductive system malignancies were excluded. (2) Imaging examinations showed new space-occupying lesion(s) at common sites of recurrence, such as lung, liver, peritoneal lymph nodes, bone, adrenal gland, and brain. Follow-up was conducted until May 2016.

Statistical analysis

Baseline characteristics were presented as mean±SD for age with two-sample t-test or n (%) for other variables with Fisher's exact test. The association between baseline characteristics and recurrence was determined using two-sample t-test or Fisher's exact text. CTC parameters were shown as counts, % of total CTCs, and range (minimum to maximum) for CTC subtypes. Changes in CTCs were classified as categorical levels and presented as n (%) by recurrent status. The association of CTCs with recurrence was evaluated using the Fisher's exact test. All statistical analyses were two-tailed and considered significant at $p<0.05$. Data were analyzed using IBM SPSS V.22 statistical software for Windows.

RESULTS

Baseline characteristics of the study subjects

Table 2 summarizes the baseline characteristics of all 47 patients (45 male/2 female), with a mean age of 48.8 years (SD=9.9) and a range of 25–66 years. During the follow-up period, 15 patients had recurrence. Baseline Edmondson stage, T stage, accumulated tumor diameter, microvascular cancer embolus, and AFP levels were significantly greater in patients with recurrence (all $p<0.05$; table 2).

CTC levels in patients with HCC receiving LTx

Analysis of CTCs in all 47 patients with HCC revealed that 70.2% were CTC-positive (table 2). For those 20 patients whose preoperative and postoperative peripheral blood CTCs were examined (group 1), 142 CTCs were identified in the preoperative samples while 151 CTCs were detected at the postoperative time point (table 3). In the preoperative samples,

Table 2 Baseline characteristics of patients with HCC by disease recurrence status following LTx (n=47)

Baseline characteristics		(n=47)	Recurrence (n=15)	No recurrence (n=32)	p Values
Age (years)	Age, mean±SD	48.8±9.9	44.9±9.7	50.6±9.6	0.069
Sex	Male	45 (95.7)	14 (93.3)	31 (96.9)	0.541
	Female	2 (4.3)	1 (6.7)	1 (3.1)	
Liver cirrhosis	No	2 (4.3)	15 (100)	30 (93.8)	1.000
	Yes	45 (95.7)	0 (0)	2 (6.3)	
Edmondson stage	I–II	1 (2.1)	0 (0)	1 (3.1)	0.027*
	II	11 (23.4)	1 (6.7)	10 (31.3)	
	II–III	20 (42.6)	5 (33.3)	15 (46.9)	
	III	15 (31.9)	9 (60)	6 (18.8)	
T stage	1–2	14 (29.8)	1 (6.7)	13 (40.6)	0.020*
	3–4	33 (70.2)	14 (93.3)	19 (59.4)	
Baseline CTCs counts (/5 mL)	≥1	40 (85.1)	12 (80)	28 (87.5)	0.664
	≥2	33 (70.2)	8 (53.3)	25 (78.1)	0.083
	≥5	26 (55.3)	6 (40.0)	20 (62.5)	0.211
Accumulated tumor diameter	≤8 cm	22 (46.8)	2 (13.3)	20 (62.5)	0.002*
	>8 cm	25 (53.2)	13 (86.7)	12 (37.5)	
Number of tumors	1	16 (34.0)	2 (13.3)	14 (43.8)	0.052
	≥2	31 (66.0)	13 (86.7)	18 (56.3)	
Maximal tumor diameter	≤5 cm	22 (46.8)	4 (26.7)	18 (56.3)	0.070
	>5 cm	25 (53.2)	11 (73.3)	14 (43.8)	
Capsule invasion	No	39 (83.0)	13 (86.7)	26 (81.3)	1.000
	Complete	8 (17.0)	2 (13.3)	6 (18.7)	
Microvascular cancer embolus	No	34 (72.3)	7 (46.7)	6 (18.8)	0.046*
	Yes	13 (27.7)	8 (53.3)	26 (81.3)	
Portal tumor embolus	No	10 (21.3)	6 (40.0)	4 (12.5)	0.054
	Yes	37 (78.7)	9 (60.0)	28 (87.5)	
Preoperative AFP	<200 µg/L	26 (55.3)	3 (20.0)	18 (56.2)	0.028*
	>200 µg/L	21 (44.7)	12 (80.0)	14 (43.8)	

Data were summarized as n (%).
*p<0.05.
AFP, alpha-fetoprotein; CTC, circulating tumor cell; HCC, hepatocellular carcinoma; LTx, liver transplantation.

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Table 3 CTC parameters by subtype in groups 1 and 2

	Group 1 (n=20 patients)				Group 2 (n=27 patients)				
	Total	Epithelial	Mixed	Interstitial		Total	Epithelial	Mixed	Interstitial
Preoperative					First postoperative time point				
CTC count	142	31	89	22	CTC count	188	36	118	34
Percentage		21.8	62.7	15.5	Percentage		19.1	62.8	18.1
Range (minimum to maximum)		0–11	0–28	0–7	Range (minimum to maximum)		0–8	0–28	0–6
Postoperative					Second postoperative time point				
CTC count	151	54	65	32	CTC count	106	27	56	23
Percentage		35.8	43	21.2	Percentage		25.5	52.8	21.7
Range (minimum to maximum)		0–21	0–22	0–6	Range (minimum to maximum)		0–3)	0–8	0–3

CTC, circulating tumor cell.

21.8% of CTCs were classified as the epithelial subtype, 62.7% as mixed, and 15.5% as the interstitial subtype. Analysis of postoperative samples revealed that 35.8% of the CTCs were classified as the epithelial subtype, 43% as mixed, and 21.2% as the interstitial subtype, suggesting a switch from a mixed subtype to either epithelial or interstitial subtypes following LTx.

For those patients whose postoperative peripheral blood CTCs were examined twice (group 2), 188 CTCs were identified at the first time point and 106 at the second time point. At the first time point, 19.1% of the CTCs were classified as the epithelial subtype, 62.8% as mixed, and 18.1% as the interstitial subtype (table 3). At the second postoperative time point, 25.5% of CTCs were classified as the epithelial subtype, 52.8% as mixed, and 21.7% as the interstitial subtype (table 3). These results suggest that the proportion of CTC subtypes may change following LTx and over the follow-up period.

Analysis of the association of CTCs with HCC recurrence following LTx

We next analyzed whether changes in the total CTCs or CTC subtypes between the first and second measurements were associated with HCC recurrence in either group. As shown in table 4, no significant associations were observed between change in total CTCs and HCC recurrence in either group (all $p>0.05$); changes in CTC subtypes were also not associated with HCC recurrence (all $p>0.05$; table 4). These results suggest CTCs (either preoperative or postoperative assessments) are not predictive of HCC recurrence following LTx.

DISCUSSION

LTx remains one of the most promising treatments for HCC,[3] with 5-year survival rates of 46.5%–60.9%.[4–6] The high recurrence rate is mainly ascribed to the incomplete

Table 4 Association of changes in CTC parameters with HCC recurrence in groups 1 and 2

	Total			Group 1			Group 2		
	Recurrence (n=15)	No recurrence (n=32)	p Values	Recurrence (n=3)	No recurrence (n=17)	p Values	Recurrence (n=12)	No recurrence (n=15)	p Values
CTC count—total			0.514			0.270			0.823
Reduction	7 (46.7)	19 (59.4)		0 (0)	9 (53.0)		7 (58.3)	10 (66.7)	
Stability	1 (6.6)	4 (12.5)		1 (33.3)	3 (17.5)		0 (0)	1 (6.6)	
Increase	7 (46.7)	9 (28.1)		2 (66.7)	5 (29.4)		5 (41.7)	4 (26.7)	
CTC count—epithelial			0.926			0.393			0.379
Reduction	4 (26.7)	11 (34.4)		0 (0)	4 (23.5)		4 (33.3)	7 (46.7)	
Stability	6 (40.0)	11 (34.4)		1 (33.3)	9 (53.0)		5 (41.7)	2 (13.3)	
Increase	5 (33.3)	10 (31.2)		2 (66.7)	4 (23.5)		3 (25.0)	6 (40.0)	
CTC count—mixed			0.818			1.000			0.821
Reduction	9 (60)	20 (62.4)		2 (66.7)	9 (53.0)		7 (58.3)	11 (73.3)	
Stability	2 (13.3)	6 (18.8)		1 (33.3)	5 (29.4)		1 (8.3)	1 (6.7)	
Increase	4 (26.7)	6 (18.8)		0 (0)	3 (17.6)		4 (33.3)	3 (20.0)	
CTC count—interstitial			0.726			0.768			0.131
Reduction	3 (20)	11 (34.4)		1 (33.3)	3 (17.6)		2 (16.7)	8 (53.3)	
Stability	7 (46.7)	12 (37.5)		1 (33.3)	9 (53.0)		6 (50)	3 (20.0)	
Increase	5 (33.3)	9 (28.1)		1 (33.3)	5 (29.4)		4 (33.3)	4 (26.7)	

Data were summarized as n (%) and analyzed using Fisher's exact test.
CTC, circulating tumor cell; HCC, hepatocellular carcinoma.

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resection of the cancer or the residual microlesions in the liver and not HBV activity given the administration of exogenous antibody against HBV. Alternatively, the required immunosuppressive treatment following LTx may compromise the killing of CTCs. Thus, we hypothesized that analysis of CTCs in patients with HCC receiving LTx may have prognostic value in identifying patients at risk for recurrence. In the present study, we showed that baseline Edmondson stage, T stage, accumulated tumor diameter, microvascular cancer embolus, and AFP levels were significantly greater in patients with recurrence. Although analysis of CTC subtypes suggested that they may change following LTx and over the follow-up period, neither preoperative nor postoperative CTC assessments were predictive of HCC recurrence following LTx.

With improvements in CTC separation and enrichment techniques, increasing studies have investigated their role as important biomarkers for the early diagnosis of cancers, evaluation of prognosis, and individualized therapy.[10–18] The CellSearch system, which employs sorting with EpCAM antibody-conjugated beads, has been approved by the US Food and Drug Administration for CTC detection in patients with metastatic breast cancer, colorectal cancer, or prostate cancer. However, only 35% of HCC cells are positive for EpCAM, a marker of epithelial cells[28]; therefore, this system is inadequate for the detection of other CTC subtypes that are detected in patients with HCC. In this study, we used the CanPatrol system, which detects epithelial markers (EpCAM and cytokeratin (CK)) and interstitial markers (vimentin and twist), and permits morphological phenotyping, cell phenotyping, and molecular phenotyping of CTCs.[23] Of the 47 patients with HCC analyzed in the present study, 70.2% were CTC-positive, which was higher than that reported in patients with liver cancer, nasopharyngeal carcinoma, breast cancer, colorectal cancer, or gastric cancer.[27] It is also higher than the 35% positive rate reported for 20 patients with HCC.[29] It is possible that the greater proportion of CTCs detected in the present study was due to the use of the CanPatrol system of detection given that some previous studies have only isolated CTCs based on EpCAM expression alone,[29] which would not detect interstitial CTCs.

Residual CTCs remain a major cause of metastasis and recurrence after resection of primary cancer,[30] and changes in CTCs may be an indicator of prognosis and recurrence in patients with cancer.[31 32] Furthermore, CTCs may undergo epithelial-mesenchymal transition, which increases their invasiveness,[33 34] and interstitial CTCs were closely related to the metabolism and resistance to chemotherapy.[24] Although we detected changes in CTC subtypes following LTx and over the follow-up period, a greater proportion of epithelial and interstitial CTCs, changes in preoperative or postoperative CTCs were not associated with HCC recurrence following LTx. This is not consistent with previous studies in which EpCAM CTCs were associated with poor prognosis[29] and early recurrence following radical hepatectomy.[35] It is possible that CTC detection may still have prognostic value in terms of PFS or OS; therefore, additional studies are required to fully examine the potential of this assay in patients with HCC.

In addition to CTCs, we also investigated baseline factors that may be related with postoperative recurrence in patients with HCC receiving LTx. Our results showed the accumulated tumor diameter, preoperative AFP, T stage, Edmondson stage, and microvascular tumor embolus were closely related to postoperative recurrence and could serve as indicators for the prediction of recurrence after LTx in patients with HCC. However, portal tumor embolus and capsule invasion were not related to postoperative recurrence, which was consistent with findings reported by Guo *et al*.[36]

The present study is limited by its small sample size from a single institution. Thus, additional large, multicenter studies are necessary to determine whether the number and subtypes of CTCs can be used as predictive factor for HCC recurrence after LTx.

In conclusion, baseline Edmondson stage, T stage, accumulated tumor diameter, microvascular cancer embolus, and AFP levels may be predictive of HCC recurrence following LTx. Although CTC subtypes may change following LTx and during the follow-up period, neither preoperative nor postoperative CTC assessments were predictive of HCC recurrence following LTx. Further large, multicenter studies are necessary to confirm the results of the present study.

Contributors SW: study concept, study design, definition of intellectual content, clinical studies, experimental studies, data analysis, statistical analysis, manuscript preparation. YZ: clinical studies, experimental studies. JL: literature research, data acquisition. FH: manuscript editing, manuscript review. JZ: guarantor of integrity of the entire study, manuscript review. All authors have read and approved the final version to be submitted.

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Competing interests None declared.

Patient consent Obtained.

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