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Relationship between circulating tumor cells undergoing EMT and short-term efficacy following interventional treatment in patients with hepatocellular carcinoma

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ABSTRACT

Objective: A growing number of studies have indicated that epithelial-mesenchymal transition (EMT) phenotypes and the number of circulating tumor cells (CTCs) are significant indicators of tumor characteristics and treatment efficacy, and thus have a broad range of potential applications in the diagnosis and treatment of malignant tumors. The value of data on CTC phenotypes and CTC counts in the diagnosis of hepatocellular carcinoma (HCC) and assessment of efficacy after comprehensive interventional therapy remains unclear.

Methods: Data of 107 patients who exhibited space-occupying lesions in the liver on enhanced CT/MRI scans at the Guangdong Provincial People's Hospital (a tertiary medical center) between August 2017 and October 2018, were retrospectively analyzed. All enrolled patients were treated with transcatheter arterial chemoembolization (TACE) combined with microwave ablation (MWA). An imFISH CTC assay was used to isolate and count CTCs with different EMT phenotypes in the patients' peripheral blood, which facilitated an analysis of the value of CTC phenotype and CTC count data in the diagnosis or treatment of HCC.

Results: The CTC count and EMT phenotypes in HCC patients were not associated with patient characteristics such as age, sex, Hepatitis B Virus (HBV)-DNA status, alcohol consumption history, Aspartate Transaminase (AST) to Platelet Ratio Index (APRI) score, Eastern Cooperative Oncology Group (ECOG) score, Child-Pugh score, alpha-fetoprotein (AFP), number and size of tumors, vascular invasion, or metastasis ($P > 0.05$). The CTC count and EMT phenotypes in HCC patients before treatment were not predictive of short-term efficacy ($P > 0.05$). Comprehensive interventional therapy reduced the total CTC count and mesenchymal CTC count ($P = 0.034$ and 0.022, respectively).

Conclusion: TACE in combination with ablation reduced the total CTC count and mesenchymal CTC count. The CTC count and EMT phenotypes may be associated with long-term efficacy.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and is the second leading cause of cancer-related deaths. This cancer type causes nearly 800,000 deaths annually, with >50% of new cases or deaths occurring in China.[1] Despite improvements in monitoring and treatment techniques,[2] due to latent liver disease and a high incidence of recurrence and metastasis after treatment, the prognosis is still poor. Pathologic biopsy is generally considered the gold standard for clinical diagnosis and decision-making by clinicians and researchers. Due to the invasion and boundaries of conventional pathologic biopsies, the biopsy specimens are unable to represent tumor heterogeneity and overall status, and do not allow for monitoring of dynamic tumor progression. Therefore, it is necessary to find a new minimally invasive or non-invasive diagnostic method to detect HCC at an early stage and to monitor the efficacy of HCC treatment. Over the recent years, a new diagnostic concept (liquid biopsy) which is non-invasive and allows for repeated analyses, has emerged and has gained substantial attention.[3,4] Circulating tumor cell (CTC) monitoring in HCC has been clinically applied owing to the rapid development of molecular detection techniques; however, the diagnostic reliability of these data is unknown. In this study we analyzed 107 patients with hepatic malignancies, and used the imFISH method to detect different CTC phenotypes and evaluate CTC counts in patients. We then analyzed the

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intrinsic relationship between CTCs and the basic characteristics of HCC to evaluate the reliability of CTC monitoring for early diagnosis and prognosis determination in patients with HCC.

Materials and methods

Ethical statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study has been approved by the Ethics Committee of the Guangdong Provincial People's Hospital, and all patients or their families have provided relevant informed consent. The ethical approval number is No. GDREC 2016437H.

Study design and clinical follow-up

This retrospective study was conducted at the Guangdong Provincial People's Hospital as a single-arm and single-center trial. Blood samples were collected for analysis within three days before interventional treatment, and retesting was conducted after six months. A Cyttel System (Cyttel, Jiangsu, China) was used to isolate and count CTCs with different phenotypes. The patients had not received treatment before enrollment. All of the enrolled patients had a confirmed diagnosis of HCC based on histologic examination or imaging criteria of the European Association for the Study of the Liver (EASL). Patients who presented with a diagnosis of another malignant tumor were excluded from this study.

Patients were treated with transcatheter arterial chemoembolization (TACE) combined with microwave ablation (MWA). All patients underwent enhanced computed Tomography (CT)/Magnetic Resonance Imaging (MRI) 1–2 months after each surgery, and the efficacy was evaluated using the modified response evaluation in solid tumors (mRECIST). The Ethical Committee of our hospital approved the study protocol, and written informed consent was obtained from all participants.

Procedure

TACE treatment

The Seldinger puncture technique was used to build a vitro channel. Hepatic angiography was performed using a RH or YASHIRO catheter, and the nutritional artery of the tumor was entered using a Termao microcatheter. Pirarubicin (50 mg), iodized oil (Guerbet, France), and a non-ionic contrast agent were emulsified together. The ratio of iodinated oil to contrast agent was 1:1, and the amount of iodinated oil was determined according to tumor size and tumor blood supply, with the maximum amount not exceeding 20 mL. The iodized oil emulsion was then pulse-injected into the nutritional artery of the tumor, until embolization was complete (i.e., the flow rate of tumor blood slowed and the nutritional artery disappeared with administration of 2–5 cardiac contrast agents). An enhanced CT scan was performed within one week after TACE to evaluate the deposition of the iodized oil.

MWA treatment

Following CT completion, data acquisition, and reconstruction, the ablation needle (ECO-100AI10, ECO) was introduced into the predetermined site under CT guidance, and the target lesion was ablated based on intra-operative CT and pre-operative planning. When the optimal insertion angle and depth were achieved, the specific power and time settings were typically 5–10 min with a 65 W (W) ablation. The duration of ablation was directly related to the quality of the surrounding

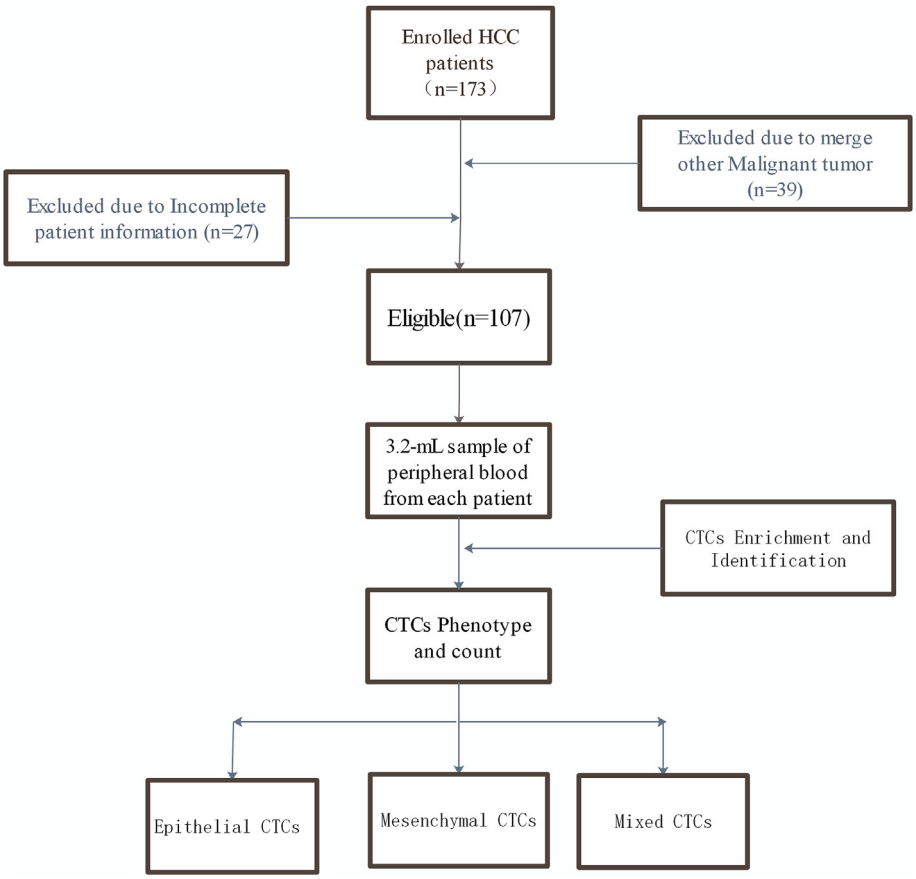

Fig. 1.

liver tissue, lesion depth, and demarcation line length. Finally, a CT scan was performed after ablation to determine the ablation range.

Detection of different phenotypes and CTC count

The Cyttel method was used to isolate and count CTCs with different phenotypes. Peripheral venous blood (3.2 mL) was collected in a BD vacutainer tube (Becton-Dickinson Company, Franklin, NJ, USA) within three days before treatment, and processed in CS1 and CS2 buffers (Cyttel) in succession. The resulting cell pellet was re-suspended in CS1 buffer, and then incubated with anti-CD45 antibody-conjugated immuno-magnetic beads (Cyttel). The white blood cells were separated by gradient centrifugation at 300g for 5 min in CS3 buffer (Cyttel). The resulting solution containing CTCs was smeared on a slide (Thermo Fisher Scientific, Franklin, NJ, USA), fixed, and dried for subsequent analysis.

The dried specimens were fixed and dehydrated, and a centromere of chromosome 8 (CEP8) probe (Cyttel) was added to the slides. The slides were placed in an automated hybridization instrument. Anti-human CD45 was added to the slides at room temperature, and the slides were mounted with mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA).

Measurements of other clinical indicators

The best method by which to determine the severity of liver fibrosis clinically is a liver biopsy, but repeated monitoring during long-term treatment is difficult due to the invasive nature of the procedure, and poor reproducibility. The 2016 APASL consensus guideline[5] and the EASL- Asociacion Latinoamericana para el Estudio del Higado (ALEH) clinical practice guideline[6] announced that the aspartate aminotransferase-to-platelet ratio index (APRI) can provide high-quality evidence in the diagnosis or exclusion of significant liver fibrosis and cirrhosis.

APRI refers to the ratio index of Aspartate Transaminase (AST) and platelet count (PLT). APRI was calculated according to the following formula: APRI = (AST/upper limit of normal (ULN)) × 100/PLT (10^9/L). The ULN for AST is 40 U/L. It is generally accepted that an APRI score >2 points indicates that an adult patient has developed cirrhosis.[7] According to previous studies, APRI has a significant correlation with the prognosis of HCC after treatment, and also has important implications for HCC surveillance in cured Hepatitis C Virus (HCV) patients.[8,9]

Statistical analysis

SPSS 20.0 statistical analysis software was used. Measurement data are expressed as the mean ± standard deviation (SD), and independent sample t-tests were used for inter-group comparisons. Numerical data are described using the number of cases or percentages, and χ^2-tests were used for comparison. The test level was set at an $\alpha = 0.05$, and a $P < 0.05$ indicated statistical significance.

Results

Patient characteristics

From August 2017 to October 2018, a total of 173 patients exhibited space-occupying lesions in the liver as per the enhanced CT/MRI scan. These patients were considered for inclusion in this study. Thirty-nine patients presented with a diagnosis of another malignant tumor and 27 patients had missing information. As a result, 107 HCC patients (102 men and 5 women) were included in this study (Fig. 1). The average age was 58.96 ± 13.24 years; details are shown in Table 1.

Among the enrolled patients, we analyzed the relationship between EMT phenotypes or CTC count and patient' characteristics, but did not detect statistical differences; the details are shown in Tables 2 and 3. All of the patients received TACE combined with MWA treatment. There were no differences among the patients in short-term efficacy at the 1st, 3rd, and 6th months following treatment; the details are shown in Table 4. After 6 months, the EMT phenotypes and CTC counts of 61 patients were reviewed. The total number of CTCs and the mesenchymal CTC counts were significantly lower than those before interventional therapy (P = 0.034 and P = 0.022, respectively); the details are shown in Table 5.

Table 1
Baseline characteristics of enrolled patients.

Characteristic	Parameter
Age (years)	58.96 ± 13.24
Sex (Male/Female)	102/5
History of hepatitis B	72 (67.3%)
HBV DNA (≥100)	41 (38.3%)
APRI (>2)	14 (13.3%)
Child-Pugh score (A/B)	91/16
BCLC stage (A/B/C)	19/53/35
ECOG PS (0/1/2)	56/43/8
Lesion characteristic (Single/<3/Multifocal)	30/21/56
Diameter of Lesion (mm)	62.14 ± 39.28
Portal vein invasion	24 (22.4%)
AFP (ng/ml)	4438.53 ± 11806.43
Total bilirubin	20.53 ± 12.34
Albumin	35.5 ± 5.21

HBV, Hepatitis B Virus; APRI, Aspartate aminotransferase to Platelet Ratio Index; BCLC, Barcelona Clinic Liver Cancer; ECOG, Eastern Cooperative Oncology Group; AFP, alpha fetoprotein.

Discussion

CTC detection method

Ashwort reported tumor cells similar to primary tumor in the

Table 2
Relationship of circulating tumor cells (CTCs) with patient demographics and baseline characteristics.

Items	Total CTC		Epithelial CTC		Mixed CTC		Mesenchymal CTC	
	≥3	<3	≥1	<1	≥1	<1	≥1	<1
Age, years								
<50	26	12	10	28	10	28	36	2
≥50	34	35	24	45	15	54	63	6
P		0.056		0.368		0.592		0.793
Sex								
Male	57	45	33	69	23	79	95	7
Female	3	2	1	4	2	3	4	1
P		0.856		0.930		0.719		0.826
HBV DNA								
<100	37	29	20	46	17	49	62	4
≥100	23	18	14	27	8	33	37	4
P		0.997		0.678		0.458		0.742
History of Alcohol Consumption								
Yes	3	1	0	4	2	2	4	0
No	57	46	34	69	23	80	95	8
P		0.792		0.399		0.496		0.562
APRI score								
≤2	52	41	29	64	20	73	86	7
>2	8	6	5	9	5	9	13	1
P		0.931		0.734		0.241		0.959
ECOG scope								
<1	34	22	19	37	9	47	53	3
≥1	26	25	15	36	16	35	46	5
P		0.311		0.616		0.062		0.613
Child-Pugh scope								
A	52	37	31	58	22	67	84	5
B	8	10	3	15	3	15	15	3
P		0.276		0.218		0.667		0.257
Albumin								
<30	8	11	3	16	3	16	16	3
≥30	52	36	31	57	22	66	83	5
P		0.176		0.099		0.574		0.299

CTC, Circulating tumor cells; HBV, Hepatitis B Virus; APRI, Aspartate aminotransferase to Platelet Ratio Index; ECOG, Eastern Cooperative Oncology Group.

Table 3
Relationship of circulating tumor cells (CTCs) with tumor characteristics.

Items	Total CTC		Epithelial CTC		Mixed CTC		Mesenchymal CTC	
	≥3	<3	≥1	<1	≥1	<1	≥1	<1
Largest nodule size								
<5 cm	33	20	18	35	11	42	49	4
≥5 cm	27	27	16	38	14	40	50	4
P		0.201		0.630		0.527		0.978
Tumor distribution								
Unifocal	14	16	7	23	6	24	28	2
2–3 tumors	11	10	9	12	4	17	17	4
Multifocal	35	21	18	38	15	41	54	2
P		0.344		0.336		0.677		0.107
AFP								
<400 ng/mL	39	29	20	48	14	54	64	4
≥400 ng/mL	21	18	14	25	11	28	35	4
P		0.725		0.488		0,370		0.656
Hepatic vein invasion								
No	56	45	30	71	22	79	94	7
Yes	4	2	4	2	3	3	5	1
P		0.909		0.150		0.276		0.935
Cheng's Classification of Portal vein invasion								
0-I	50	33	25	58	18	65	77	6
II-III	10	14	9	15	7	17	22	2
P		0.106		0.494		0.446		0.856
Metastasis								
No	45	37	24	58	16	66	74	8
Yes	15	10	10	15	9	16	25	0
P		0.651		0.313		0.088		0.234
BCLC stage								
A + B	42	30	20	52	13	59	66	6
C	18	17	14	21	12	23	33	2
P		0.500		0.203		0.063		0.927

CTC, Circulating tumor cells; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha fetoprotein.

peripheral blood of patients with cancer in 1869, thus proposing the concept of CTCs for the first time. CTCs refer to tumor cells derived from the primary tumor or metastases, which enter into the blood circulation during tumor formation and progression.[10] CTCs can be divided into epithelial, mixed, and mesenchymal types, based on whether or not epithelial-mesenchymal transition (EMT) has occurred.[11] The detection of CTCs from a blood sample in patients with HCC can be a very powerful non-invasive method for the early diagnosis of HCC, and for the evaluation of efficacy of administered therapy. Because the number of CTCs in peripheral blood is low, a higher sensitivity and specificity of CTC detection is needed. The Cellsearch system, in which the detection of CTCs is based mainly on the detection of the epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK) molecule, is the only technical device currently marketed for CTC testing. This device was approved by the United States Food and Drug Administration (FDA) in 2004. EpCAM and CK are down-regulated during EMT concurrent with tumor invasion and metastasis, leading to false-negative detection of CTCs by the Cellsearch system. It is unlikely that this approach detects CTCs which have undergone EMT.[12] Conventional positive and negative selection-based CTC isolation methods which are not based on EpCAM detection may miss metastasis-relevant CTCs which undergo EMT, and also yield samples with a lower purity. Some studies have proposed that compared to EpCAM-based Cellsearch CTC detection, *in situ* karyotypic identification performed by interphase fluorescence *in situ* hybridization (iFISH) has a higher detection rate.[13,14] In the present study, to evaluate the clinical value of CTC detection, a novel integrated negative enrichment and immunostaining-fluorescence hybridization (imFISH) platform was applied to analyze CTCs in patients with HCC. The system showed good performance in serum, which suggests that imFISH may be an effective tool for clinical use in patients with HCC.

Table 5
Changes in circulating tumor cell (CTC) counts after treatment compared to the counts at preoperative conditions in 61 patients.

Items	Preoperative	Six months after treatment	P
Total CTC	2.6 ± 1.52	2.31 ± 1.50	0.034
Epithelial CTC	0.38 ± 0.58	0.28 ± 0.55	0.321
Mixed CTC	0.34 ± 0.60	0.21 ± 0.413	0.172
Mesenchymal CTC	2.21 ± 1.45	1.84 ± 1.31	0.022

CTC, Circulating tumor cell.

Relationship between CTCs and patient characteristics

Numerous studies indicate that monitoring CTCs may have a wide range of potential applications in the early diagnosis of malignant tumors, assessment of conditions, selection of treatment methods, and monitoring of prognosis. Due to the high negative rate of AFP in HCC, the combined use of CTCs and AFP detection may enhance the sensitivity of early diagnosis of HCC.[15–17]

The CTC count or the EMT phenotypes and CTC count in HCC patients were not associated with patient characteristics such as age, sex, HBV-DNA status, history of alcohol consumption, APRI score, ECOG scope, Child-Pugh score, AFP, number and size of tumors, vascular invasion, or metastasis ($P > 0.05$; Tables 2 and 3).

The relationship between CTCs and therapeutic effects in HCC

Metastatic HCC is a leading cause of cancer deaths worldwide, and CTCs are critical components in the intra- or extra-hepatic metastatic process in HCC. CTCs are predominantly epithelial when released into the circulatory system, but switch to an EMT-activated phenotype due to the activation of several signaling pathways during hematogenous transit.

Table 4
Relationship of circulating tumor cells (CTCs) with short-term efficacy of TACE combined with MWA in patients.

Items	Total CTC			Epithelial CTC			Mixed CTC			Mesenchymal CTC		
	≥3	<3	P	≥1	<1	P	≥1	<1	P	≥1	<1	P
1st												
OR	41	39	0.083	25	55	0.841	18	62	0.716	75	5	0.684
DC	53	46	0.063	31	68	0.718	23	76	0.910	92	7	0.574
3rd												
OR	39	35	0.293	12	21	0.496	18	56	0.725	69	5	0.979
DC	53	46	0.063	30	69	0.250	23	76	0.910	92	7	0.574
6th												
OR	39	33	0.568	26	46	0.167	14	58	0.168	67	5	0.764
DC	54	41	0.653	33	62	0.064	20	75	0.112	89	6	0.199

CTC, Circulating tumor cell; OR, Objective Response Rate; DC, Disease control rate; CR, Complete Response; PR, Partial Response; SD, Stable Disease.
Evaluating the relationship between CTC counts and short-term efficacy in patients was performed by using the Chi-square test. $P < 0.05$ was considered significant. No difference was observed among patients in the 1st, 3rd, and 6th months.
(OR: Objective Response Rate = CR + PR, DC: disease control rate = CR + PR + SD).

Huaman established a syngeneic mouse model of HCC and showed that CTCs exhibit distinct characteristics from primary tumor-derived cells; in the above mentioned study, a greater migration of CTCs compared to primary tumor-derived cells was observed, in addition to decreased E-cadherin and increased SLUG and fibronectin expression.[18] Previous research has suggested that CTCs in hepatic veins and peripheral circulation prognosticate post-operative lung metastasis and intrahepatic recurrence, respectively.[19] Mixed CTCs may be a vital factor for intrahepatic metastasis, and mesenchymal CTCs have the potential to be a predictor of extrahepatic metastasis.[20]

In this study, neither the level of total CTCs nor the EMT phenotypes in HCC patients before treatment was predictive of short-term efficacy ($P > 0.05$). Comprehensive interventional therapy may reduce the total number of CTCs and mesenchymal CTCs ($P = 0.034$ and 0.022, respectively), thus affecting the long-term efficacy in patients (Tables 4 and 5). Based on previous research, HCC patients with positive peripheral mesenchymal CTCs have a higher risk of early recurrence, and thus peripheral mesenchymal CTC presence could be a potential biomarker in HCC prognosis monitoring.[21] A reduction in the total number of CTCs and mesenchymal CTCs may indicate effective control of tumor progression. This finding could be one of the most effective ways to monitor therapeutic outcomes. Ye demonstrated that post-operative CTC counts (>2 and > 5) and changes in CTC counts may be independent prognostic indicators for progression-free survival (PFS) in patients with HBV-related HCC, with a change in the number of CTCs showing better predictive performance.[22] Based on multivariate Cox regression analysis, CTC count was found to be an independent predictor of overall survival ($P = 0.049$) and PFS ($P = 0.007$) in patients treated with chemoembolization.[23]

Surgical liver resection is associated with an increase in CTC count, and an increased post-operative CTC count is associated with a worse prognosis in patients with HCC.[24] Considering the risk of tumor recurrence and metastasis, clinicians must develop appropriate treatment plans for patients. TACE combined with ablation is a widely accepted treatment for patients with HCC according to the National Comprehensive Cancer Network (NCCN), and has been shown to lead to better outcomes for patients.[25,26]

This study has some limitations. First, it was a single-center retrospective study with the small sample size, which may have resulted in unexpected deviations during data extraction and analysis. Second, the potential ability of the CTC count and EMT phenotypes to predict long-term survival of HCC patients as yet remains unclear. Long-term follow-up is needed to assess the predictive performance of the CTC count and EMT phenotype parameters.

Patient consent

Witten informed consent was obtained from patients for publication of these case reports and any accompanying images.

Declaration of competing interest

The authors declare that they have no conflicts of interests to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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None.

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