Original Article

Circulating tumor cells and CXCR4 in the prognosis of hepatocellular carcinoma

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Background: This study was to determine circulating tumor cells (CTCs) and the expression of CXC chemokine receptor type 4 (CXCR4) in primary hepatocellular carcinoma (HCC) and the relationships with prognosis.

Methods: We used an advanced CanPatrol™ CTC-enrichment technique to collect CTCs for isolation and characterization from blood samples. The RNA in situ hybridization (RNA-ISH) method, which is based on branched DNA (bDNA) signal amplification technology, was used to determine the expression of CXCR4 according to epithelial-mesenchymal transition (EMT) markers in 99 patients with primary liver cancer in blood samples pre-operatively. The relationship between the EMT markers and HCC was determined.

Results: The positive rates of CTCs and CXCR4 were 89.9% and 58.8%, respectively. CTCs were positively correlated with the Barcelona clinic liver cancer (BCLC) staging, tumor diameter and number, envelope, microsatellite damage, portal vein thrombosis, alpha-fetoprotein (AFP), and hepatitis B DNA, and negatively correlated with Edmondson grade. There were significant differences in the expression of CXCR4 between interstitial CTCs and mixed CTCs. A total of 99 patients underwent CTCs testing prior to surgery. The tumor-free survival time of HCC patients with interstitial CTCs <1 (13.3 months) was significantly longer than patients with interstitial CTCs ≥1 (5.0 months) pre-operatively.

Conclusions: CTC-positivity was shown to be associated with HCC and can be used as an independent prognostic factor for HCC. High CXCR4 protein expression was more common in mixed CTCs.

Keywords: Hepatocellular carcinoma (HCC); circulating tumor cells (CTCs); chemokine receptor; prognosis

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Introduction

Hepatocellular carcinoma (HCC) is a major health problem worldwide. there were more than 782,000 cases diagnosed annually, and 50% in China alone (1). There are currently many treatments for HCC, liver transplantation and surgical resection are the preferred treatment methods (2). But a significant number of patients cannot achieve the expected effect due to post-operative metastasis and recurrence. Microvascular invasion (MVI) can usually be clearly diagnosed in postoperational pathology. And metastases are usually found by chance during resection, both of which are associated with significantly worse prognosis (3). Hence, it is necessary to determine the risk factors for HCC recurrence and the markers for continuous monitoring of anti-tumor response before and after surgery.

Circulating tumor cells (CTCs) is an integral part of "liquid biopsy" and has great potential to change the current treatment modality in the cancer field (4). CTCs are derived from solid tumors and are associated with hematogenous metastasis (5). Therefore, analyzing the level of CTCs has clinical guiding significance. In lung cancer, CTCs exist in dynamic stages of primary tumor detaching and disseminating into the blood (6). For liver cancer patients, overall survival (OS) tended to be poorer in patients with CTCs (7).

The CXC chemokine receptor type 4 (CXCR4) is a specific receptor for the chemokine stromal cell-derived factor-1 (CXCL12). In general, CXCR4 helps activate the immune system and stimulates cell movement, but when the signal that activates the receptor is not properly modulated, CXCR4 accelerates the progression and spread of cancer (8).

CTCs and CXCR4 are indicators associated with prognosis of patients after tumor resection. The relevance of these two indicators has been reported for many tumors, but the relevance between peripheral CTCs and tumor tissue CXCR4 in HCC patients has rarely been reported (9). The relationships between peripheral CTCs, HCC stage, and CXCR4 expression on tumor tissues were analyzed in this study.

Methods

Patients

The clinical information of 99 patients with a diagnosis of primary liver cancer that were treated in the Hepatobiliary Surgery Department of our hospital between March 2016 and January 2017 were prospectively analyzed. There were 85 males and 14 females with an age range of 20–72 years, and an average age of 46.19±10.12 years (see *Table 1* for Baseline data of 99 patients enrolled in CTCs). One hundred thirty detections of CTCs were performed; 99 patients agreed to perform CTCs detection once pre-operatively. Statistical analysis was based on pre-operative/pre-therapy data. The clinical diagnosis standard for the patients was in accordance with the 2015 edition of *The Standardized Case Diagnosis Guidelines of Primary Liver Cancer*. None of the patients had received targeted therapy, radiotherapy, chemotherapy, and/or intervention therapy before surgery or treatment, and all of the patients accepted the detection of CTCs in peripheral blood before surgery. Patients with other systemic tumors and autoimmune diseases were excluded. This study was reviewed and approved by the hospital Ethics Committee (LW2019059), and all of the patients signed informed consent.

Collection, processing, and tumor cell enrichment of peripheral blood samples

EDTA anti-coagulation blood collection tubes were used to collect 5 ml of peripheral blood samples from patients with liver cancer pre-operatively and mixed by inversion. Fifteen milliliters of red blood cell lysate were added to the peripheral blood samples, mixed well, and the cell pellet was resuspended with PBS. The residual cell pellet was fixed with formaldehyde at a final concentration of 4%. The cells in the suspension were filtered using a CanPatrol™ system, and the fixed cells were transferred to a filter tube containing a filter, and the cells were filtered onto an 8 μM filter using a vacuum pump. The filtered cell filter samples were fixed at room temperature using 4% formaldehyde.

Multiple RNA in situ analysis

The cells entrapped by the filter were typed using a multiplex RNA probe and the level of the cell CXCR4 gene expression was detected. According to the target gene type of the probe, specific capture probe epithelial biomarker probes (EpCAM and CK8/18/19), interstitial biomarker probes (vimentin and twist), and a leukocyte marker (CD45) were added for hybridization (see *Table 2* for probe sequences).

First, the cells were pre-treated with a permeabilizing agent and a protease. Subsequently, three sets of RNA probes were added, incubated, and branched DNA (bDNA) signal amplification probes were added. Thus,

Table 1 Baseline data of 99 patients enrolled in CTCs

Variables (n)	Patient population (n=99)
Sex (male/female)	85/14
Age (<45/≥45 years)	44/55
BCLC staging (A/B/C staging)	29/32/38
Edmondson staging (high/medium/low-undifferentiated)	35/42/22
Number of tumors (equal to 1/>1)	65/34
Size of tumors (≤5/>5 cm)	22/77
Envelope (complete/incomplete)	64/35
Microvascular tumor thrombus (with/without)	47/52
Portal vein tumor thrombus (with/without)	34/65
Child-Pugh classification (A/B/C level)	94/5/0
AFP ≤400/>400 ng/mL	38/61
Hepatitis B DNA (<5×10^{e2}/≥5×10^{e2})	36/63
CTCs <1/CTCs ≥1	48/51

CTC, circulating tumor cell; BCLC, Barcelona clinic liver cancer; AFP, alpha-fetoprotein.

Table 2 Capture probe sequences

Gene	Sequence (5'-3')
EpCAM	TGGTGCTCGTTGATGAGTCA
	AGCCAGCTTTGAGCAAATGA
	AAAGCCCATCATTGTTCTGG
	CTCTCATCGCAGTCAGGATC
	TCCTTGTCTGTTCTTCTGAC
	CTCAGAGCAGGTTATTTCAG
CK8	CGTACCTTGTCTATGAAGGA
	ACTTGGTCTCCAGCATCTTG
	CCTAAGGTTGTTGATGTAGC
	CTGAGGAAGTTGATCTCGTC
	CAGATGTGTCCGAGATCTGG
	TGACCTCAGCAATGATGCTG

Table 2 (*continued*)

Gene	Sequence (5'-3')
CK18	AGAAAGGACAGGACTCAGGC
	GAGTGGTGAAGCTCATGCTG
	TCAGGTCCTCGATGATCTTG
	CAATCTGCAGAACGATGCGG
	AAGTCATCAGCAGCAAGACG
	CTGCAGTCGTGTGATATTGG
CK19	CTGTAGGAAGTCATGGCGAG
	AAGTCATCTGCAGCCAGACG
	CTGTTCCGTCTCAAACTTGG
	TTCTTCTTCAGGTAGGCCAG
	CTCAGCGTACTGATTTCCTC
	GTGAACCAGGCTTCAGCATC
Vimentin	GAGCGAGAGTGGCAGAGGAC
	CTTTGTCGTTGGTTAGCTGG
	CATATTGCTGACGTACGTCA
	GAGCGCCCCTAAGTTTTTAA
	AAGATTGCAGGGTGTTTTCG
	GGCCAATAGTGTCTTGGTAG
Twist	ACAATGACATCTAGGTCTCC
	CTGGTAGAGGAAGTCGATGT
	CAACTGTTCAGACTTCTATC
	CCTCTTGAGAATGCATGCAT
	TTTCAGTGGCTGATTGGCAC
	TTACCATGGGTCCTCAATAA
CD45	TCGCAATTCTTATGCGACTC
	TGTCATGGAGACAGTCATGT
	GTATTTCCAGCTTCAACTTC
	CCATCAATATAGCTGGCATT
	TTGTGCAGCAATGTATTTCC
	TACTTGAACCATCAGGCATC
CXCR4	ACTGCCTTGCATAGGAAGTT
	GGTTGACTGTGTAGATGACA
	AGGATGAGGACACTGCTGTA
	TAGCGGTCCAGACTGATGAA
	CAACATAGACCACCTTTTCA
	AATAGTCAGCAGGAGGGCAG
	CGTTGGCAAAGATGAAGTCG
	TATCTGTCATCTGCCTCACT

Table 3 Sequences for the bDNA signal amplification probes

Items	Function [copies]	Sequence [5'-3']	Complement [copies]
bDNA probes for EpCAM and CK8/18/19	Capture probe tail [1]	CTACAAACAAACAATATT	Preamplifier leader [1]
	Preamplifier repeat [5]	CGCAGCCTCAGCC	Amplifier leader [1]
	Amplifier repeat [5]	CCCAGACCCTACC	Label probe [1]
bDNA probes for vimentin and twist	Capture probe tail [1]	CTTCTCAATAACTAACAT	Preamplifier leader [1]
	Preamplifier repeat [5]	GACGGTCGGCGTT	Amplifier leader [1]
	Amplifier repeat [5]	GTCACCGCTCCAC	Label probe [1]
bDNA probes for CD45	Capture probe tail [1]	GTAAAAAGAAAGGTATAA	Preamplifier leader [1]
	Preamplifier repeat [5]	AATTATACATCTC	Amplifier leader [1]
	Amplifier repeat [5]	GAAATGAATGAAT	Label probe [1]
bDNA probes for CXCR4	Capture probe tail [1]	CTTTATACCTTTCTTTCA	Preamplifier leader [1]
	Preamplifier repeat [5]	GCGCGCTGTAGGG	Amplifier leader [1]
	Amplifier repeat [5]	AGGCGAGGGGAGA	Label probe [1]

bDNA, branched DNA.

there were four labeled fluorescent proteins (see *Table 3* for probe sequences), as follows: Alexa Fluor 594 [for labeled epithelial biomarker probes (EpCAM and CK8/18/19)]; Alexa Fluor 488 [interstitial biomarker probes (vimentin and twist)]; Alexa Fluor 750 [for labeling the leukocyte marker (CD45)]; and Alexa Fluor 647 (for labeling CXCR4) in combination with an RNA probe. Finally, the nuclei were stained with DAPI and observed under a fluorescence microscope (Olympus, Tokyo, Japan). The red and green fluorescent signal points represented the expression of epithelial and mesenchymal genes on CTCs, respectively. White signal dots represented the leukocyte marker (CD45) gene expression. Purple signal dots represented CXCR4 gene expression (see *Figure 1*).

Clinical pathologic indicators

Intrahepatic recurrent cancer and extrahepatic metastases were found within 2 years post-operatively on re-examination and considered to be early tumor recurrences. The diameter of the tumor refers to the maximum diameter of the tumor. MVI referred to tumor tissues connected to or filling the vascular lumen on observation with a microscope. The tumor capsule referred to a clear demarcation between tumor and surrounding tissues on observation with unaided eyes. The surrounding tissues were oppressed with tumor and a fibrous capsule. Non-enveloping refers to an unclear boundary between the tumor and the surrounding tissue. The Edmondson-Stein grade standard was used to evaluate the degree of differentiation of liver cancer. The Barcelona clinic liver cancer (BCLC) was used as the staging standard for liver cancer.

Follow-up

Patients were asked to undergo follow-up evaluations every month for 3 months, every 2 months for the following 6 months, and every 3 months for the next 6 months.

The following tests were obtained during follow-up evaluations: blood tests, including a routine blood examination, liver and kidney function tests, alpha-fetoprotein (AFP), and electrolytes; and imaging tests, including chest radiography, abdominal ultrasonography, and computed tomography/magnetic resonance imaging. Recurrence was defined as new HCC lesions present on computed tomography or magnetic resonance imaging with or without abnormal AFP levels. The primary outcome in our study was progression-free survival (PFS). The endpoint for disease-free survival (DFS) was HCC recurrence and death.

Statistical analysis

Statistical analysis of the data was performed using

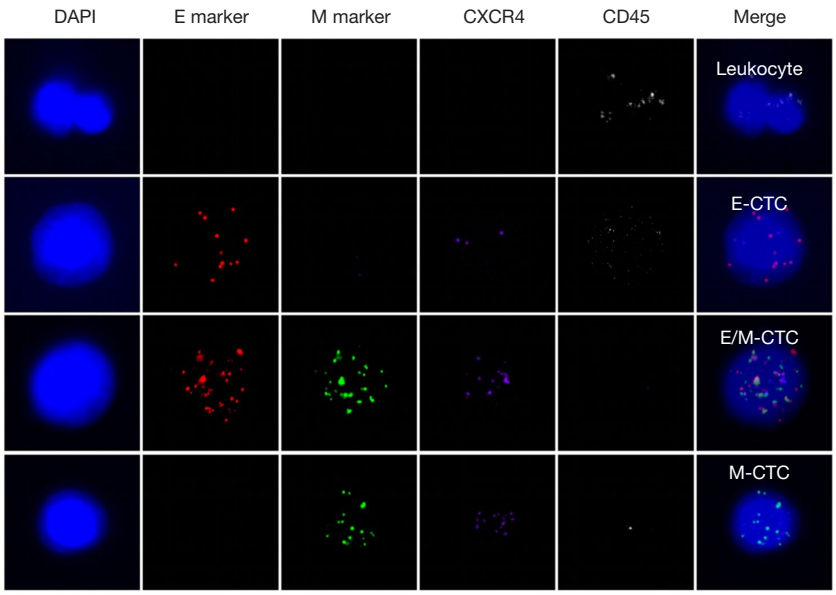

Figure 1 Detection and classification of CTCs using EMT markers. The red and green fluorescent signal points represented the expression of epithelial and mesenchymal genes on CTC, respectively. White signal dots represented the leukocyte marker (CD45) gene expression. Purple signal dots represented CXCR4 gene expression. CTC, circulating tumor cell; EMT, epithelial-mesenchymal transition.

SPSS22.0. Measurement data are expressed as the $\bar{x}\pm s$, and *t*-tests were used for the treatment of the data. A χ^2 test was used to compare the difference in enumeration data and the difference was statistically significant at a P<0.05. The Kaplan-Meier method was used to calculate the PFS.

Results

Correlation analysis of CTCs and clinical pathologic indices

The positive rate of CTCs was 89.9%. The relationship between CTCs and various clinical indicators of liver cancer was based on the CTC-positive rate, number of CTCs, types of CTCs, and the proportion of interstitial CTCs. All of the CTCs detection results were analyzed from the pre-operative results, with a focus on the clinical correlation between CTCs and BCLC stage, Edmondson classification, Child-Pugh classification, tumor diameter, tumor number, capsule invasion, MVI, surgical margins, portal vein tumor thrombus, liver cirrhosis, and AFP, alanine transaminase (ALT) levels, hepatitis B DNA, and HBsAg levels. A significant correlation existed between BCLC stage, Edmondson classification, tumor diameter, tumor number, capsule invasion, portal vein tumor thrombus, hepatitis B DNA and the total number of CTCs and distribution of CTCs types, the correlations were positive and the Edmondson classification had a negative correlation (P<0.05). No correlation existed with MVI, surgical margins, liver cirrhosis, Child-Pugh classification, HBsAg levels, and ALT levels (P>0.05), as shown in *Table 4*.

Correlation between BCLC stage of liver cancer and number and types of CTCs

Logistic regression analysis of all the BCLC patient data showed that there was a positive correlation (P=0.021). There was no correlation between epithelial CTCs and BCLC. There was a positive correlation between the number of mixed CTCs and stage (P=0.033). There was a positive correlation between the number of interstitial CTCs and stage (P<0.001); shown in *Figure 2*.

Correlation between CXCR4-positive expression and type of CTCs

There was a significant difference between CXCR4 expression in various types of CTCs. The epithelial CTCs did not generally express CXCR4, while the mixed CTCs mostly expressed CXCR4. The number of interstitial CTCs

Table 4 Correlation analysis between CTCs and every clinical liver cancer index

Spearman's rho	N	Value	CTCs positive or not	CTCs total number	Epithelial	Mixed	Interstitial	Interstitial CTCs proportion
BCLC stage 0-A/B/C	N=99	r	0.248	0.221	–0.032	0.265	0.419	0.407
		P value	0.017*	0.021*	0.764	0.033*	<0.001**	<0.001**
Edmondson stage I/II/III	N=99	r	–0.252	–0.374	–0.443	–0.271	–0.235	–0.097
		P value	0.019	<0.001**	<0.001**	0.011	0.028*	0.369
Tumor number (single/multiple)	N=99	r	–0.011	0.159	–0.039	0.126	0.375	0.272
		P value	0.917	0.132	0.71	0.236	<0.001**	0.009**
Tumor diameter (≤5/>5 cm)	N=99	r	0.148	0.109	–0.130	0.210	0.215	0.189
		P value	0.158	0.300	0.217	0.045*	0.040*	0.071
Envelope (complete/incomplete)	N=99	r	0.171	0.226	0.051	0.261	0.316	0.229
		P value	0.108	0.033	0.638	0.014*	0.003**	0.031*
Portal vein tumor thrombus (no/yes)	N=99	r	0.121	0.246	0.125	0.266	0.284	0.278
		P value	0.251	0.018*	0.236	0.010*	0.006**	0.007**
AFP (normal/abnormal)	N=99	P value	0.083	0.193	0.224	0.192	0.206	0.132
		P value	0.441	0.070	0.035*	0.071	0.053	0.218
Hepatitis B-DNA (no/yes)	N=99	r	0.096	0.299	0.251	0.215	0.039	–0.103
		P value	0.379	0.005**	0.020*	0.046*	0.720	0.345

A significant correlation was suggested when *, P<0.05 (2-tailed) or **, P<0.01 (2-tailed). CTC, circulating tumor cell; BCLC, Barcelona clinic liver cancer; AFP, alpha-fetoprotein.

expressing CXCR4 was similar to the number of interstitial CTCs not expressing CTCs, as shown in *Table 5*.

Subsistence analysis

A total of 99 patients underwent CTCs testing prior to surgery [see *Table 6* for Comparison of baseline data of interstitial CTCs <1 and ≥1 (classification data)], all of them had follow-up information related to disease recurrence and 72 had disease progression. We divided the CTCs into group A (<5) and group B (≥5), the epithelial and mixed CTCs into group A1 (<3) and group B1 (≥3), and the interstitial CTCs into group A2 (<1) and group B2 (≥1). Pre-operatively, there were 40 patients in group A and 59 in group B. The median PFS was 15.8 and 5.2 for groups A and B, respectively. There was no significant difference in PFS between the two groups (P=0.097). Pre-operatively, there were 63 patients in epithelial CTCs group A1 and 36 in group B1. The median PFS was 7.0 and 6.0 for group A1 and B1, respectively. There was no significant difference in PFS between the two groups (P=0.692). Pre-operatively, there were 48 patients in interstitial CTCs group A2 and 51 patients in group B2. The median PFS was 13.3 and 5.0 for group A2 and group B2, respectively. Group A2 had a significantly longer PFS than group B2 (P<0.05; *Figure 3*).

Discussion

Radical resection of HCC as a routine treatment has made the 5-year survival rate of small and large HCC increase to 50–60% and 30–40%, respectively, but a significant number of patients still die of liver cancer recurrence and metastasis after surgery (9). Tumor metastasis involves tumor cell shedding from the tumor, entering the bloodstream, and transfer to a distant place (10). CTCs represent various types of tumor cells present in the peripheral blood. CTCs that have left the primary lesion and entered the blood circulation (11) are considered to be important factors in the recurrence and metastasis of malignant tumors (12). After a thorough investigation, CTCs have been shown

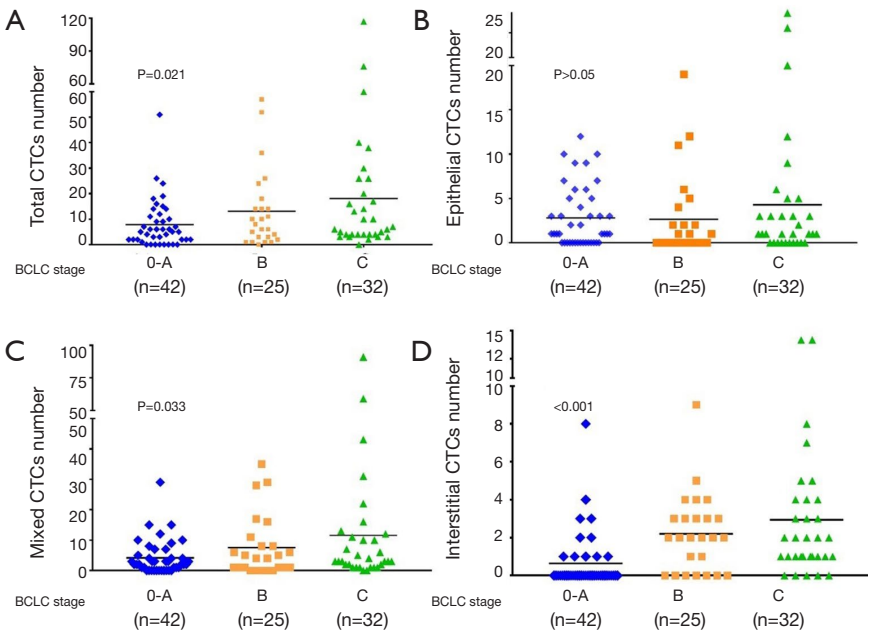

Figure 2 Correlation between BCLC stage of liver cancer and number and types of CTCs. (A) Liver cancer BCLC stage and CTCs total number; (B) liver cancer BCLC stage and epithelial CTCs number; (C) liver cancer BCLC stage and mixed CTCs number; (D) liver cancer BCLC stage and interstitial CTCs number. BCLC, Barcelona clinic liver cancer; CTC, circulating tumor cell.

Table 5 Correlation between CXCR4 positive expression and CTCs typing

CTCs typing	Cells number	CXCR4 expression		P value
		Negative (%)	Positive (%)	
Epithelial	75	50 (66.7)	25 (33.3)	<0.001
Mixed	206	63 (30.6)	143 (69.4)	
Interstitial	59	27 (45.8)	32 (54.2)	
Total	340	140 (41.2)	200 (58.8)	

CTC, circulating tumor cell.

to have great significance in cancer diagnosis, treatment, evaluation of prognosis, and individualized treatment (13). Improved Can Patrol CTC enrichment technology has divided CTCsnto three subtypes according to EMT markers (epithelial, interstitial, and mixed types) (14). The results showed that more interstitial CTCs were detected in metastatic lesions. In a study of breast cancer (15), interstitial CTCs were predominant in blood samples from patients with aggressive breast cancer types. In the current study, CTCs were detected in 99 liver cancer patients pre-operatively. CTCs are classified into different types. It was found that the positive rate of interstitial CTCs increased with the increase in BCLC stage. At the same time, interstitial CTCs and the positive rates of interstitial CTCs were the same as the total number of CTCs, which were significantly correlated with high-risk relapse indicators of HCC, such as tumor diameter, number, envelope, portal vein tumor thrombus, and AFP and hepatitis B DNA levels. This finding further indicated that CTCs that express EMT markers, i.e., interstitial and mixed CTCs, were greater in number and metastatic.

It has been shown that the positive rate of CTCs in peripheral blood of patients with liver cancer are positively correlated with the tumor stage (BCLC and TNM stage), and the positive rate of CTCs in patients with combined thrombus and extrahepatic metastasis is higher (16). Xu

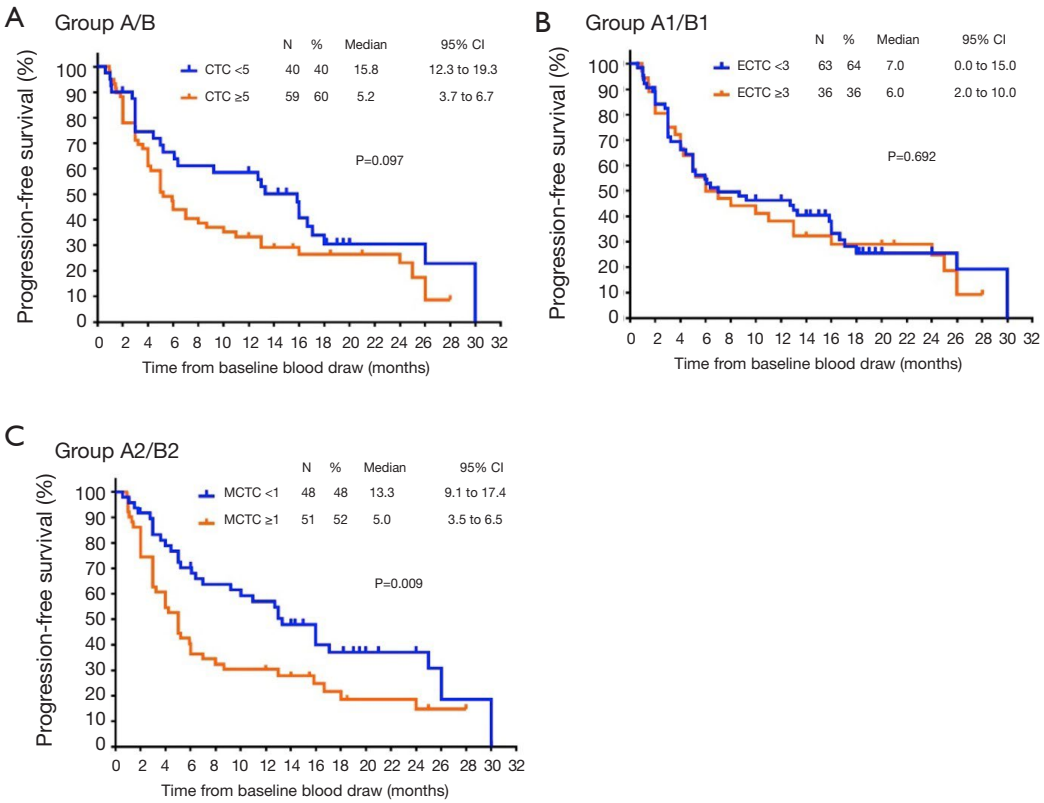

Figure 3 Preoperative CTCs number/type and PFS of patients. CTCs were divided into group A (<5) and group B (≥5), the epithelial and mixed CTCs into group A1 (<3) and group B1 (≥3), and the interstitial CTCs into group A2 (<1) and group B2 (≥1). CTC, circulating tumor cell; PFS, progression-free survival.

et al. (17) reported 85 HCC patients with ASGPR and their ligands, and found that 80% with early or liver cancer <2 cm had CTCs in the peripheral blood. Our findings were consistent with the Xu *et al.* study (17), further clarifying the role of CTCs in the guidance of follow-up individualized treatment for different stages of liver cancer. If CTCs can be used in routine examinations, it will be beneficial in the early diagnosis and treatment of HCC patients, thus prolonging the DFS and overall survival. Previous studies have suggested that portal vein tumor thrombi are the source of tumor metastases (18). There is a close relationship between CTCs and portal vein tumor thrombi. The positive rate of CTCs in peripheral blood in patients with portal vein tumor thrombi is significantly higher than patients without portal vein thrombosis (19,20). Vona *et al.* (21) used Cell Search System (CSS) to detect CTCs in 44 patients with liver cancer, and the detection rate was 52%. Xu *et al.* (17) used reverse transcription-PCR (RT-PCR) to detect CTCs in 85 HCC patients and reported that the detection rate of CTCs was significantly correlated with the existence of portal vein tumor thrombi. Our study was consistent with the above findings. We further found that interstitial CTCs and the types of CTCs that contain a high proportion of interstitial cells have a significant correlation with portal vein thrombosis, further indicating that among CTCs, interstitial CTCs have an important role in tumor chemotaxis and metastasis.

There is an abundance of research results involving the correlation between CTCs and AFP. Nel *et al.* (22) found that AFP mRNA is 75% sensitive and 71% specific by detecting EpCAM + CTC-specific gene expression. Schulze *et al.* (23) reported that the AFP levels of liver cancer patients with EpCAM + CTC patients were >400 ng/mL. Xu *et al.* (17) detected the expression of MACE-1, SSX1, and CPF11 mRNA in peripheral blood CTCs of 85 patients using RT-PCR, and found that 65.9% of liver cancer patients contained at least one of the above gene mRNA species, while the three CTC antigen gene

Table 6 Comparison of baseline data of CTCs interstitial <1 and ≥1 (classification data)

Type	Sex (male/female)	Age (<45/≥45 years)	BCLC staging (A/B/C staging)	Number of tumors (equal to 1/>1)	Size of tumors (≤5/>5 cm)	Envelope (complete/incomplete)	Envelope invasion (complete/incomplete)	Microvascular tumor thrombus (with/without)	Child-Pugh classification (A/B/C level)	Portal vein tumor thrombus (with/without)	AFP ≤400/>400 ng/mL	Hepatitis B (<5×10^{e2}/≥5×10^{e2})
Interstitial CTCs<1	41/7	23/25	17/15/16	36/12	13/35	16/19/13	33/15	20/28	46/2/0	14/34	20/28	16/32
Interstitial CTCs ≥1	44/7	21/30	12/17/22	29/22	9/42	19/23/9	31/20	27/24	47/3/0	20/31	18/33	20/31
χ^2	0.015	0.455	1.845	3.607	1.274	1.276	0.687	1.260	0.170	1.107	0.425	0.370
P value	0.903	0.500	0.397	0.058	0.259	0.528	0.407	0.262	0.680	0.293	0.515	0.543

CTC, circulating tumor cell; BCLC, Barcelona clinic liver cancer; AFP, alpha-fetoprotein.

mRNA detection rate were not significantly correlated with serum AFP levels. Our study confirmed the correlation between epithelial CTCs and AFP, but did not fully prove the relationship between CTCs and AFP. Therefore, a large sample, multicenter study is needed to clarify the relationship between CTCs and AFP in patients with HCC.

CXCR4 and its receptor, CXCL12, has been shown to be closely related to the development and metastasis of various tumors, and the relationship between the high expression of the CXCR4 gene and CTCs has been found in a number of tumors (24). Mego *et al.* (25) showed that a relationship between breast cancer CTCs and CXCR4, and revealed the role of CXCR4-SDF-1 in the mobilization and transport of endothelial CTCs. However, in the HCC studies, the data did not match with other tumors. The expression of CXCR4 in tumors was with no significant difference (26) or even lower (27) compared with normal tissues by immunohistochemistry or PCR analysis. Recently, Kaemmerer *et al.* (28) and other studies found that CXCR4 was strongly expressed in the tumor blood vessels of liver cancer, and its CXCR4 expression was associated with poor prognosis. Yu *et al.* (29) showed that peripheral MMP26 + CXCR4 + were liver cancer CTCs, and considered that CXCR4 could be used as a marker for detecting CTCs. Our previous laboratory study confirmed that the "pathways in cancer" in gene expression profile analysis of HCCs with very high CTCs levels and those with very low CTCs levels revealed ranked first with respect to the differentially expressed genes. CXCR4 was shown to play an important part in seven cancer-related biological processes, some of which were metastasis-related biological process (e.g., positive regulation of cell invasion and migration, tumor angiogenesis, negative regulation of apoptosis, and chemotaxis) (30). We observed that most of the genes involved in these metastasis-related biological processes were up-regulated. Thus, some compensatory dissemination/metastasis mechanisms may exist in HCCs which may result in a high CTC count and M-CTCs percentage. Our study further found that the expression of CXCR4 in different types of CTCs was significantly different. The expression rate of CXCR4 in interstitial CTCs (interstitial and mixed CTCs) was >50%. This is in agreement with our previous laboratory findings.

To this end, we did tumor-free survival follow-up in 99 HCC patients who underwent CTCs before surgery, and found that with the exception of the number of pre-operative CTCs, the types of CTCs all affected the tumor-free survival time. Although there is no statistical difference

in the total number of CTCs, the trend was favorable. In terms of the total number of CTCs, 5 CTCs/7.5 mL of peripheral blood were counted as the boundary value, and the tumor-free survival was significantly longer in patients with <5 CTCs compared to >5 CTCs. This affirmed the importance of CTCs as a post-operative prognostic indicator for liver cancer patients. The greater the expression of pre-operative peripheral blood CTCs, the poorer the prognosis. At the same time, in the research of different types of CTCs, we found that interstitial CTCs, with more invasion and metastatic potential, can be more accurate in the assessment of tumor-free survival time for liver cancer patients, which is consistent with other CTCs related to malignant tumors, metastatic breast cancer, colon cancer, and prostate cancer (31-33). Because of the small number of patients and limitation of the follow-up, only 22 patients had survival-related follow-up information, and total survival were not included in the study. Therefore, in future studies, large sample sizes and patient follow-up information will be refined for further validation.

In summary, peripheral CTC positivity is related to the recurrence and prognosis of HCC and can be used as an independent prognostic factor for HCC. At the same time, high CXCR4 protein expression was more common in mixed CTCs. The monitoring of peripheral blood CTCs should be a dynamic observation process combined with the characteristics of liver cancer to find the discipline of the changes in peripheral blood CTCs and guide clinical treatment. We bring new hope for accurately judging the clinical staging of liver cancer, guiding individualized treatment, and evaluating the prognosis.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.01.14). The authors have no conflicts of interest to declare.

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 was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was reviewed and approved by the hospital Ethics Committee (LW2019059), and all of the patients signed informed consent.

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