Original Article

Significance of circulating tumor cells in the portal vein regarding metastases and vascular invasion in hepatocellular carcinoma patients

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Background: Vascular invasion is an important risk factor of poor prognosis in hepatocellular carcinoma (HCC) patients. The detection of circulating tumor cells (CTCs) in the blood is direct evidence of tumor presence. There are few reports on CTCs and metastasis and vascular invasion of HCC. The purpose of this study was to analyze the significance of CTCs in the portal vein regarding metastases and vascular invasion in HCC patients.

Methods: A total of 104 HCC patients diagnosed and treated in Zhengzhou University People's Hospital were enrolled. Surgery was performed in 60 individuals. Portal vein blood samples were collected before treatment for CTCs detection. We used the isolation by size of epithelial tumor cells (ISET) and fluorescence in situ hybridization (FISH) to enrich and classify CTCs from blood samples. The patients were divided into metastasis and nonmetastasis groups according to the metastasis status before treatment. Differences in clinical indicators such as alpha-fetoprotein (AFP) levels, tumor size, CTCs count, and macrovascular tumor thrombus between the two groups were analyzed as well as the associations of CTCs count with the above indicators. For individuals with postoperative pathology, the relationship between CTCs counts and microvascular invasion (MVI) was analyzed.

Results: The amounts of portal vein CTCs were higher in patients with metastases compared with the nonmetastases group (20 *vs.* 7; z=3.795; P<0.001). Multivariate logistic regression analysis showed that the CTC count was a risk factor for HCC metastasis [odds ratio (OR) =1.044; 95% CI: 1.011–1.079]. The sensitivity and specificity of CTC count in predicting HCC metastasis were 82.93% and 52.38%, respectively. CTC count was significantly correlated with tumor size (r_s=0.308; P=0.001), vascular invasion (z=4.211; P<0.001), and MVI (z=12.763; P=0.002). A threshold CTC count of seven showed the most significant power for predicting metastasis.

Conclusions: Vascular invasion positivity was closely related to HCC metastasis. Portal vein CTC count before treatment was correlated with vascular invasion and could be considered one of the factors affecting HCC metastasis. However, the ability of CTC count was limited in predicting HCC metastasis due to insufficient specificity.

Keywords: Portal vein; circulating tumor cell (CTC); hepatocellular carcinoma (HCC); metastasis; vascular invasion

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Introduction

Hepatocellular carcinoma (HCC) represents a malignant tumor type with high morbidity and mortality worldwide (1,2). Hepatitis B virus (HBV) infection is a major risk factor for HCC. The 5-year survival rate of HCC patients is only between 8.37% to 12.1% due to recurrence and metastasis (3,4). Vascular invasion is the basis for tumor cell entry into the circulation as well as metastasis and is a critical risk factor for HCC recurrence and poor survival (5,6).

Macrovascular invasion of HCC often presents as vascular tumor thrombus (VTT), which can be found on imaging. Portal vein tumor thrombus is the most common form, occurring in about 10% to 60% of patients at the time of diagnosis (7). Microvascular invasion (MVI) appears as micrometastatic HCC emboli within the vessels detected by pathological examination. MVI in the portal vein is a potential source of intrahepatic metastasis and an indication of adjuvant chemotherapy after curative resection (5,7). However, MVI can only be determined based on surgical specimens. Therefore, use of serum biomarkers is expected to be a noninvasive method for MVI detection.

Previous research showed that tumor cells circulate in the bloodstream at an early stage, even before the primary tumor is clinically established (8). Therefore, the detection of circulating tumor cells (CTCs) in the blood is direct evidence of tumor presence. CTCs carry comprehensive tumor information and have stem cell characteristics, which are associated with poor prognosis (9-11). CTCs contribute to tumor metastasis through the epithelial-to-mesenchymal transition (EMT) (12). Abnormal angiogenesis and angioarchitecture in the whole process from hepatitis B fibrosis, cirrhosis to HCC are the ananatomical basis for promoting the entry of CTCs into the blood circulation (13,14). Many studies have demonstrated the association between the presence of either pre- or postoperative CTCs and an increased risk for HCC recurrence (15,16). HCC patients with positive peripheral mesenchymal CTCs have a more serious risk of early recurrence (17). At present, CTCs are recognized as the main source for HCC recurrence and metastasis after radical excision and are considered the key to understanding critical mechanisms of tumor metastasis, prognosis estimation, and treatment evaluation (10,15,18-20).

HCC spread to other regions of the liver via portal vein invasion, while metastasis to other organs mainly via hepatic veins (6,21). CTC counts in different vessels of an HCC patient are significantly dissimilar (22). CTCs in peripheral blood are considered a useful diagnostic and prognostic biomarker in HCC (20,23). However, CTC amounts in peripheral blood are low due to apoptosis, attacks of the immune system, the destruction of hemodynamic shear forces, and capillary bed obstruction in the lung (24,25). CTCs count is higher in the hepatic vein compared with the peripheral and portal veins (22). However, CTC numbers in different hepatic vein branches overtly differ due to tumor location and the multicentric or intrahepatic metastatic status of lesions in HCC patients. The presence of portal vein invasion is associated with intrahepatic metastasis and constitutes a negative prognostic factor for HCC (5). However, CTC count in the portal vein and its associations with metastasis and vascular invasion have not been reported previously. Therefore, we hypothesized that portal vein CTCs may have some influence on metastasis and vascular invasion of HCC. The purpose of this study was to investigate the associations of portal vein CTCs with metastasis and vascular invasion in patients with HCC. We present the following article in accordance with the STARD reporting checklist (available at https://dx.doi.org/10.21037/jgo-21-734).

Methods

Subjects and specimens

The current prospective study was approved by the Clinical Ethics Committee of Zhengzhou University People's Hospital (No. 2020199). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Subjects newly diagnosed with HCC between January 2019 and December 2020 were consecutively enrolled (Appendix 1; the flow chart of the study is in Appendix 2—Figure S1). To ensure the homogeneity of samples, all HCC patients in the cohort had a history of chronic HBV infection. All subjects were confirmed using pathology or clinical criteria according to the Treatment of Primary Liver Cancer in China criteria (2017 Edition). Inclusion criteria were: (I) HCC with chronic HBV infection and (II) no prior

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treatment. Exclusion criteria were: (I) a history of other malignancies, (II) main portal vein embolus or cavernous transformation preventing blood sample collection from the portal vein, and (III) declining enrolment. All subjects provided signed informed consent.

All subjects underwent thorough examinations before treatment, including laboratory tests, abdominal ultrasound, contrast-enhanced ultrasound, abdominal computed tomography (CT), and/or plain and contrast-enhanced magnetic resonance imaging (MRI) and chest CT. All imaging diagnoses were made independently by two specialists. If the results were inconsistent, consensus would be reached after discussion with experts. Tumor staging was performed according to the Barcelona Clinic Liver Cancer (BCLC) staging system. In subjects who underwent resection, all tumor specimens were confirmed using pathology. Edmondson-Steiner (E-S) grading was carried out according to tumor cell differentiation, and MVI stages were defined according to the number of microemboli and the distance from the tumor.

Serum alpha-fetoprotein (AFP) levels, tumor size, VTT, and tumor stages were recorded for each subject. The E-S grade and MVI were recorded for subjects who underwent surgery. Tumor size and VTT were determined using abdominal ultrasound, CT, and MRI. Tumor size was indicated by the maximum diameter of the primary tumor. For multifocal lesions, the maximum diameter was taken as the tumor size. The diagnosis of VTT was based on embolus enhancement in the arterial phase on contrast-enhanced CT or MRI. Based on enhanced CT or MRI, which is currently the clinically recognized standard for the diagnosis of metastasis, multiple intrahepatic lesions, peritumor subfoci, and VTT were considered as metastasis.

Blood sample collection

In subjects who underwent surgery, portal vein blood was collected via direct puncture using a 23-gauge needle before tumor resection. In those administered radiofrequency ablation or palliative treatment, ultrasound-guided percutaneous transhepatic portal vein puncture using a 20-gauge needle was performed within 1 week before treatment. Five milliliters of venous blood from each subject were collected using a 5-mL ethylenediaminetetraacetic acid vacutainer tube (BD, K2E). After the percutaneous puncture procedure, the puncture site was pressurized for 5 minutes and wrapped with a binding belt. The subject was instructed to remain supine for 24 hours. Blood samples were stored at 4–8 ℃ and processed within 6 hours of collection.

CTCs isolation and quantitation

The isolation by size of epithelial tumor cells (ISET) method, which has been used to enrich CTCs in studies involving various cancers and were demonstrated to be more sensitive than CellSearch (26,27), was used to isolate blood cells. Then, mononuclear cells and CTCs were isolated using density gradient centrifugation with the separation medium (HLSM1077, MultiSciences, Hangzhou, China). CTCs were identified based on immunofluorescent staining. After separating possible tumor cells, the phenotype of CTCs was further assessed using fluorescence in situ hybridization (FISH). FISH is a microscopic technique in which specific DNA sequences tagged with fluorophores are used to detect target genes and identify their localization within a cell with good sensitivity and reproducibility, which has been used to detect CTCs in many malignancies (28). The specific probes used for FISH included epithelial (EpCAM, CK8/18/19), mesenchymal (twist, vimentin), and leukocyte (CD45) probes. The messenger RNAs (mRNAs) of target cells were hybridized with a specific probe, which was labeled with a specific fluorescent protein. High-power fluorescence microscopy was performed to distinguish and count the fluorescent signals.

Under a fluorescence microscope, effectiveness was defined only when more than seven single fluorescent signals were detected. White blood cells, which showed purple fluorescence, were not counted as CTCs. Red and green, fluorescent signals represented epithelial and mesenchymal CTCs, respectively, and epithelial-mesenchymal mixed CTCs showed a mixture of red and green, fluorescent signals (*Figure 1A-1C*). Multicellular groups of CTCs (≥3 cells) were considered CTC clusters. The number of CTCs was obtained by counting fluorescent signals in a single cell. The investigators who performed CTC testing were blinded to the clinical indicators and metastasis of HCC patients and vice versa (the flow chart of the study is in Appendix 2—Figure S1).

Samples that could not be examined immediately were stored in cell-freezing medium at –80 ℃. The stored cell samples were analyzed within 14 days.

Statistical analysis

We conducted a descriptive analysis of demographic

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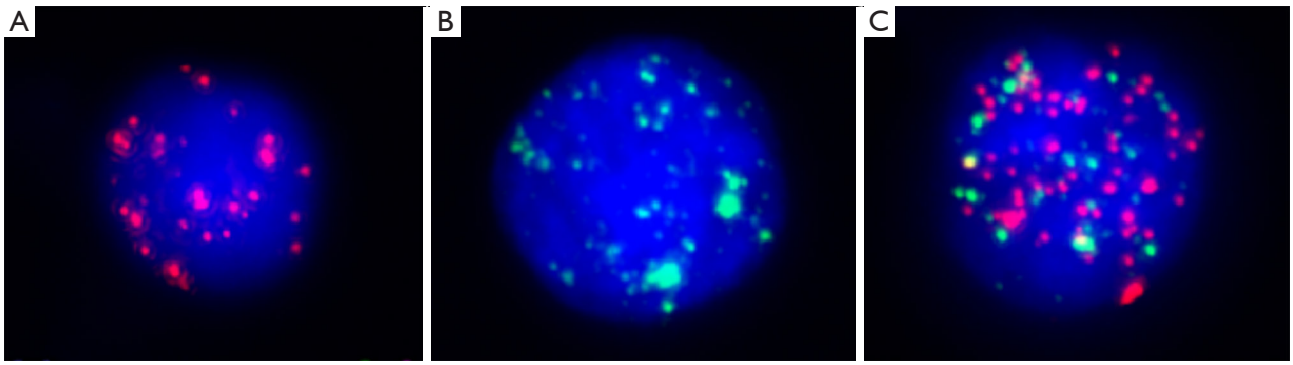

Figure 1 Red fluorescent signals represented epithelial CTCs (A); green fluorescent signals represented mesenchymal CTCs (B); and mixture of red and green fluorescent signals represented epithelial-mesenchymal mixed CTCs (C). CTCs, circulating tumor cells.

characteristics and indicators. A univariate comparative analysis of these variables was performed by grouping based on the metastasis status prior to treatment. Continuous variables are presented as means ± standard deviations (SDs) or medians and interquartile ranges (IQRs). Differences between the non-metastasis and metastasis groups were assessed using independent samples *t*-tests or Mann-Whitney U tests, depending on data normality. Pearson's chi-square tests were performed for binary and categorical variables, and Mann-Whitney U tests for ordinal variables. In addition, Mann-Whitney U tests or Spearman's rank correlations were used to explore the associations of clinical indicators with CTC count when appropriate. Then, receiver operating characteristic (ROC) curves and interactive dot diagrams were generated to evaluate the predictive value of CTC count in HCC metastasis, and the cut-off point value was determined. At last, multivariate logistic regression model was used to identify risk factors for and analyze the influence of CTCs on HCC metastasis.

Data missing indicators after repeated checks were not included in the statistical analysis. A P value <0.05 was considered as indicating statistical significance. All statistical analyses and illustrations were performed with SAS 9.1 (SAS Institute Inc., Cary, NC USA), MedCalc 18.2 (MedCalc Software Ltd., Ostend, Belgium), and GraphPad Prism 7.0 (Graph Pad Software Inc., La Jolla, CA, USA).

Results

Patient characteristics

A total of 104 subjects were enrolled in this study, including 88 males and 16 females, with a median age of 56.4±11.44 years. Forty-one subjects had intrahepatic or/and extrahepatic metastases at the time of diagnosis. Of the VTT cases diagnosed using imaging, one was inferior vena cava invasion, six were hepatic vein invasion, and the remaining were portal vein branch invasion.

Sixty of these subjects underwent resection with subsequent adjuvant therapy appropriate to their conditions, and portal vein blood was obtained via direct puncture intraoperatively. Thirty-seven subjects received palliative therapy, e.g., radioactive iodide implantation, transcatheter arterial chemoembolization (TACE), or chemotherapy to alleviate symptoms and improve quality of life. The remaining seven cases rejected any treatment. Ultrasound-guided percutaneous transhepatic portal vein puncture was performed in the above 44 subjects. After percutaneous puncture, 12 subjects complained of mild abdominal pain, but none of them suffered severe complications.

Differences between the metastasis and nonmetastasis groups

Before treatment, the subjects were divided into the metastasis and nonmetastasis groups based on their metastasis status. Both subfoci surrounding the primary tumor and multifocal lesions were defined as metastases. Gender and age were evenly distributed between the two groups, while CTC count in the portal vein, tumor size, serum AFP levels, and VTT were significantly different in the metastatic group compared with the nonmetastatic group. Moreover, the distributions of Child-Pugh and BCLC grades were significantly different between the two groups (*Table 1*).

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Table 1 Univariate analysis of demographic characteristics and clinical indicators of the metastasis and metastasis-free groups

Items	Metastasis-free (n=63)	Metastasis (n=41)	Statistics	P value
Gender, n (%)			0.529	0.467[a]
Male	52 (82.5)	36 (87.8)		
Female	11 (17.5)	5 (12.2)		
Age, mean ± SD	57.38±11.49	54.51±13.01	−2.522	0.14[b]
CTCs, median [IQR]	7 [3, 19]	23 [12, 40]	−4.903	<0.001[c]
Epithelial tumor cells (%), median [IQR]	0 [0, 10.53]	0 [0, 5.71]	−0.353	0.724[c]
Mixed tumor cells (%), median [IQR]	100 [83.33, 100]	91 [81.35, 97.25]	−1.555	0.12[c]
Mesenchymal tumor cells (%), median [IQR]	0 [0, 0]	2.94 [0, 9.17]	−4.036	<0.001[c]
Tumor size, median [IQR]	5 [3.5, 8]	12.7 [6.3, 15.4]	−4.779	<0.001[c]
AFP, n (%)			6.211	0.013[a]
>7	34 (54.0)	32 (78.0)		
<7	29 (46.0)	9 (22.0)		
Child-Pugh grade, n (%)			−3.984	<0.001[c]
A	49 (77.8)	16 (39.0)		
B	12 (19.0)	20 (48.8)		
C	2 (3.2)	5 (12.2)		
BCLC grade, n (%)			−6.3	<0.001[c]
A	16 (25.4)	2 (4.9)		
B	39 (61.9)	7 (17.1)		
C	7 (11.1)	20 (48.8)		
D	1 (1.6)	12 (29.3)		
VTT, n (%)			60.157	<0.001[a]
Positive	2 (3.2)	31 (75.6)		
Negative	61 (96.8)	10 (24.4)		
Treatment, n (%)				0.004[d]
Untreated	1 (1.6)	6 (14.6)		
Conservative treatment	19 (30.2)	18 (43.9)		
Excision	43 (68.2)	17 (41.5)		

[a], Pearson's chi-square test; [b], independent samples *t*-test; [c], Mann-Whitney U test; [d], Fisher's test. SD, standard deviation; CTCs, circulating tumor cells; IQR, interquartile range; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; VTT, vascular tumor thrombus.

Phenotypes of CTCs

CTCs in most subjects (99/104, 95.2%) showed a mixed epithelial and mesenchymal phenotype. There was no significant difference in the proportion of mixed phenotype between the two groups. Moreover, CTCs in 42.3% (44 cases) of subjects showed an epithelial phenotype, while 37.5% (39 cases) had a mesenchymal phenotype. The proportion of CTCs with a mesenchymal phenotype in the metastatic group was higher than that of the non-

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Table 2 Univariate analyses of CTCs count and clinical parameters

Items	Frequency	CTCs, M [P_{25}, P_{75}]	Statistics	P value
AFP			z=0.973[a]	0.331
>7	66	15.5 [5, 25.5]		
<7	38	9 [4.75, 23.75]		
VTT			z=4.211[a]	<0.001
Positive	33	23 [13, 44.5]		
Negative	71	8 [4, 19]		
Tumor size			r_s=0.308[c]	0.001
BCLC grade			H=26.688[a]	<0.001
A	18	5 [1.75, 7.5]		$P_{A,B}$=0.133[b]; $P_{A,C}$<0.001[b]; $P_{A,D}$<0.001[b]; $P_{B,C}$=0.022[b]; $P_{B,D}$=0.043[b]; $P_{C,D}$=0.999[b]
B	46	10 [3, 21]		
C	27	24 [8, 42]		
D	13	20 [14, 52.5]		
Child-Pugh grade			H=4.877[a]	0.087
A	65	10 [4, 21]		
B	32	16.5 [6, 32]		
C	7	17 [13, 68]		

[a], Mann-Whitney U test or Kruskal-Wallis H test; [b], Bonferroni correction for multiple tests; [c], Spearman's rank correlation analysis. CTCs, circulating tumor cells; AFP, alpha-fetoprotein; VTT, vascular tumor thrombus; BCLC, Barcelona Clinic Liver Cancer.

metastatic group. There were no significant differences in the proportions of CTCs with epithelial and mesenchymal phenotypes between the two groups. No CTC cluster was found.

CTCs and clinical indicators

The relationships between the number of CTCs and clinically relevant indicators were examined. The results showed that the number of CTCs was significantly correlated with tumor size, VTT, and BCLC grade, while there were no significant correlations between the CTC count and serum AFP levels and Child-Pugh grade (*Table 2*).

Clinical indicators related to metastasis

A multivariate logistic regression model was used to identify risk factors for HCC metastasis. The results of the model's hypothesis test showed that the P values of the likelihood ratio and Wald scores were all less than 0.0001, indicating that the model was valid. Deviance and Pearson's goodness of fit statistics showed P values of 0.9999 and 0.9958, respectively, suggesting that the model had a good fitting effect on the data as a whole. The max-rescaled *R*-square of the model was 0.7758, suggesting that 77.58% of the variation in the dependent variable could be explained by the variation of the four independent variables in the model.

Multivariate logistic regression analysis showed that the risk factors for HCC metastasis were CTC count, tumor size, AFP levels, and VTT, whose odds ratios (ORs) and 95% CIs were 1.073 (1.008–1.142), 1.199 (1.033–1.391), 5.885 (0.979–35.361), and 73.99 (11.59–472.322), respectively (*Table 3*). The standardized regression coefficients of CTC count, tumor size, AFP levels, and VTT were 0.8101, 0.4997, 0.4728, and 1.1098, respectively, suggesting that the factors affecting HCC metastasis were VTT, CTC count, tumor size, and AFP levels in sequence.

Diagnostic accuracy of the CTC count

The area under the ROC curve (AUC) of the CTC count for the diagnosis of HCC metastasis was 0.785 (95% CI:

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Table 3 Multivariate logistic regression analysis of risk factors for HCC metastasis

Items	Regression coefficient	Standardized regression coefficient	S.E.	P value	Wald chi-square	OR	95% CI
Constant	–5.778	–	1.324	<0.001	19.056	0.003	–
CTC count	0.07	0.8101	0.032	0.027	4.8871	1.073	(1.008, 1.142)
Tumor size	0.181	0.4997	0.076	0.017	5.7293	1.199	(1.033, 1.391)
AFP	1.772	0.4728	0.915	0.053	3.753	5.885	(0.979, 35.361)
VTT	4.304	1.1098	0.946	<0.001	20.7075	73.99	(11.59, 472.322)

HCC, hepatocellular carcinoma; OR, odds ratio; CTC, circulating tumor cell; AFP, alpha-fetoprotein; VTT, vascular tumor thrombus.

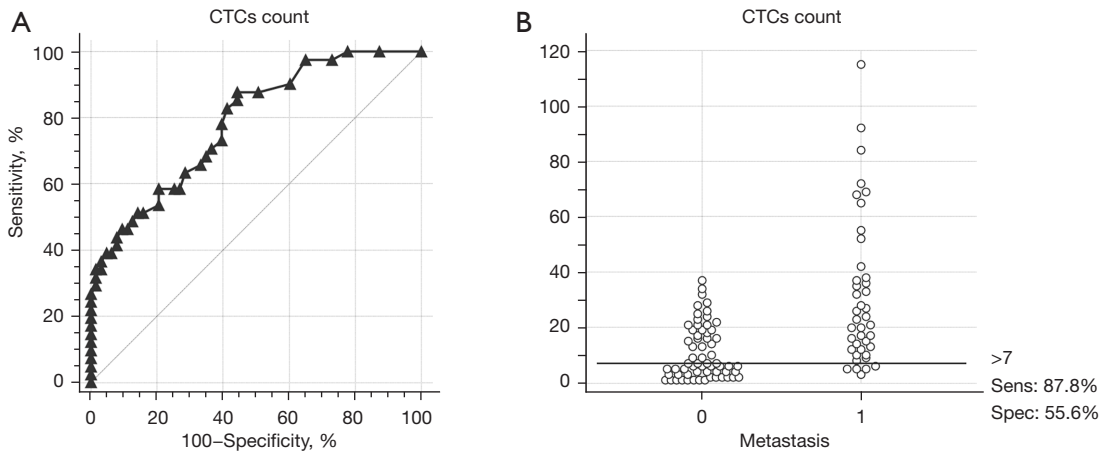

Figure 2 Diagnostic accuracy of CTCs count for the diagnosis of hepatoma metastasis. (A) ROC curve for CTCs count in the diagnosis of hepatoma metastasis. (B) Interactive dot diagram of CTCs count for diagnosis of hepatoma metastasis. ROC, receiver operating characteristic; CTCs, circulating tumor cells.

0.694–0.86, P<0.001); the corresponding sensitivity and specificity were 87.8% and 55.56%, respectively, with the best cut-off point of seven and a Youden index of 0.4336 (*Figure 2A,2B*).

Associations of CTC count with pathological indicators

Of the 60 cases who underwent tumor resection, 38 were MVI positive, including 26 and 12 MVI grades 1 and 2, respectively. Univariate analysis showed that CTC count in portal vein blood was significantly associated with E-S grade and MVI. However, according to pairwise comparisons of E-S grades, only the difference between grades I and IV was statistically significant. In addition, the difference between MVI levels 1 and 2 was not significant (*Table 4*).

Discussion

Portal vein pressure and blood flow change gradually during the progression from hepatitis B fibrosis to cirrhosis and HCC. Firstly, impaired hepatic sinusoids increase intrahepatic vascular resistance (29), and the persistent inflammatory and fibrogenic processes promote significant angiogenesis and abnormal angioarchitecture (14,30). Pressure changes and vascular disruption lead to the formation of spontaneous intrahepatic arterioportal shunt at the level of the trunk, sinusoids, or peribiliary venules (13). Secondly, hypervascularity and marked vascular abnormalities, such as arterialization and sinusoidal capillarization, are commonly associated with HCC, although following diverse causes of liver damage (31). Tumor vessels in HCC are mainly leaky

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Table 4 Univariate analysis of CTCs count and pathological parameters

Items	Frequency	CTCs, M [P_{25}, P_{75}]	Statistics	P value
E-S grade			H=8.563	0.036[a]
I	8	5 [1.75, 6.75]		$P_{I,II}$=0.132[b]; $P_{I,III}$=0.129[b]; $P_{I,IV}$=0.049[b]; $P_{II,III}$=0.999[b]; $P_{II,IV}$=0.999[b]; $P_{III,IV}$=0.999[b]
II	32	15 [4.5, 22.75]		
III	16	19.5 [3.5, 28]		
IV	4	33 [9.5, 78.25]		
MVI			H=13.883	0.001[a]
0	22	4.5 [2, 14.5]		$P_{0,1}$=0.086[b]; $P_{0,2}$=0.001[b]; $P_{1,2}$=0.151[b]
1	26	15 [6.75, 25.75]		
2	12	28 [17.5, 50]		

[a], Kruskal-Wallis H test; [b], Bonferroni correction for multiple tests; CTCs, circulating tumor cells; E-S, Edmondson-Steiner; VTT, vascular tumor thrombus; MVI, microvascular invasion.

and tortuous, with unpaired arterialized neovascularization. In addition, hepatic vasculature invasion is also one of the characteristics of HCC (7). Therefore, increased intrahepatic vascular resistance, chaotic blood flow in tumor and liver parenchyma, and vascular invasion of tumor cells may provide a passage for CTCs into the portal vein.

Portal vein CTCs and clinical indexes

The portal vein CTC counts of patients with metastasis were higher than those of individuals without metastasis, and the number of CTCs was positively correlated with tumor diameter, BCLC grade, and VTT. A threshold CTC count of seven showed the most significant power in predicting metastasis. Based on the results of multivariate logistic regression, keeping other factors unchanged, the risk of HCC metastasis increased by 1.199 times for each cm increase in tumor diameter and 1.073 times for each CTC increase. The risk of metastasis increased 73.99 times with positive VTT. These results confirmed that VTT is closely associated with HCC metastasis, and vascular invasion may be more severe in patients with larger tumors and/or metastasis. In addition, the number of CTCs in the portal vein may be related to the shedding of cancer cells on the embolus surface. Therefore, the number of portal vein CTCs before treatment was correlated with vascular invasion and could be considered a risk factor for HCC metastasis, corroborating previous studies (10,23). The number of CTCs was associated with BCLC grade, but pairwise comparison between grades revealed no significant differences in the number of CTCs between grade A and B and between grade C and D. This may be explained by the fact that some factors in BCLC grading are not necessarily relevant to CTC count.

However, CTC count is limited in its ability to determine HCC metastasis. Firstly, the OR for the CTC count's association with presence of metastasis was only 1.073, indicating a weak influence of CTCs. Moreover, the specificity of CTC count for the diagnosis of HCC with pretreatment metastases was only 55.56%, with a Youden's index of only 0.4336, suggesting that CTC count is not accurate in predicting HCC metastasis and could not be used as an independent diagnostic criterion (32).

Phenotype of portal vein CTCs

The EMT process is considered the driving force of local invasion (33). Through the EMT process, CTCs acquire the ability to resist apoptosis, with increased blood dissemination and lymphatic vessel invasion (33,34). In addition, the levels of twist and/or vimentin in EMT are correlated with portal vein tumor embolus (35).

In this study, most CTCs were of a mixed epithelial and mesenchymal phenotype, which is considered a vital factor in intrahepatic metastasis (36). In addition, the proportion of CTCs with a mesenchymal phenotype was significantly higher than that of those with an epithelial phenotype in the metastatic group, which is thought to be associated with shorter progression-free survival (11). However, none of the three subtypes of CTCs had an impact on tumor prognosis in this study, which is quite different from previous reports

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(11,36). The possible reason is that the subtypes were excluded from the regression model due to their associations with other risk factors. In addition, sampling error is also a possible reason.

Portal vein CTCs and MVI

Both VTT and MVI, which represent macrovascular invasion and MVI, respectively, are important risk factors for early recurrence and poor prognosis in HCC (5). Preoperative prediction of MVI has an impact similar to that of the detection of macrovascular invasion and is a major factor in deciding on treatment strategy (37). The relationship between VTT and HCC metastasis was confirmed. Since MVI can only be confirmed pathologically, we analyzed the pathological data of 60 subjects who had HCC resection. The results showed that the CTC count in portal vein blood was associated with MVI and tumor cell differentiation. This means that the worse the tumor cell differentiation, the higher the E-S grade, the higher the degree of tumor malignancy, and the higher the number of CTCs. However, according to pairwise comparisons within E-S grades, only the difference between grades I and IV was statistically significant, and the difference between MVI levels 1 and 2 was not significant. Therefore, portal vein CTC count could indicate tumor cell differentiation and MVI to some extent but did not correspond to E-S and MVI grades.

The main limitation of this study is that not all subjects underwent tumor resection, such that pathological parameters such as MVI could not be examined for the whole group like VTT. However, the data of the 60 cases assessed confirmed the relationship between CTC count and MVI and provided a basis for further research. Besides, the amount of CTCs in blood sample is small and dynamic. ISET and FISH technology were used to enrich and characterize CTCs in this study. The effectiveness of these two methods had been proved in many literatures (26-28). Moreover, repeated sampling to obtain more cells is possible (38). A prospective, multicenter randomized clinical trial should be designed to further validate the prognostic significance of CTCs.

To summarize, vascular invasion positivity was closely related to HCC metastasis. The number of portal vein CTCs before treatment was correlated with vascular invasion and can be considered a risk factor for HCC metastasis. However, the ability of the CTC count to determine HCC metastasis was limited due to insufficient specificity. Moreover, the CTC count in portal vein blood was associated with clinical and pathological grades but did not correspond to their specific grades.

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Footnote

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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 current prospective study was approved by the Clinical Ethics Committee of Zhengzhou University People's Hospital (No. 2020199). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). All subjects provided signed informed consent.

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Appendix 1

Sample size estimation

The sample size was estimated based on tests for one ROC curve. The hypothesis of this study was that CTCs count can effectively distinguish whether HCC has metastasis, that is, the area under ROC curve (AUC) is greater than 0.5. According to the meta-analysis results of previous studies (32), the area under ROC curve of CTCs count was 0.93. When the value of significant level (α) was 0.05 (two-sided), the power of the test (1–β) value was 0.9, and the sample allocation ratio was 1:1, power analysis and sample size (PASS) 11.0 software estimated that at least 7 cases with metastasis and 7 cases without metastasis need to be recruited.

Since there might be many factors influencing HCC metastasis, the accuracy of the CTCs count in diagnosing HCC metastasis may be overestimated due to confounding factors when univariate analysis was performed. The AUC was set at 0.8 after a pre-test and expert panel discussion. All else equal, other parameters being unchanged, at least 17 patients with metastasis and 17 patients without metastasis should be recruited. Considering the influence of confounding factors and the principle of feasibility, we planed to double the sample size to ensure the inclusion of samples can obtain sufficient test efficacy. Furthermore, given that the shedding rate was set at 10%, more than 38 subjects need to be recruited in each group.

Finally, 104 subjects were actually enrolled in this study, of which 41 subjects developed metastasis and 63 subjects did not.

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Appendix 2

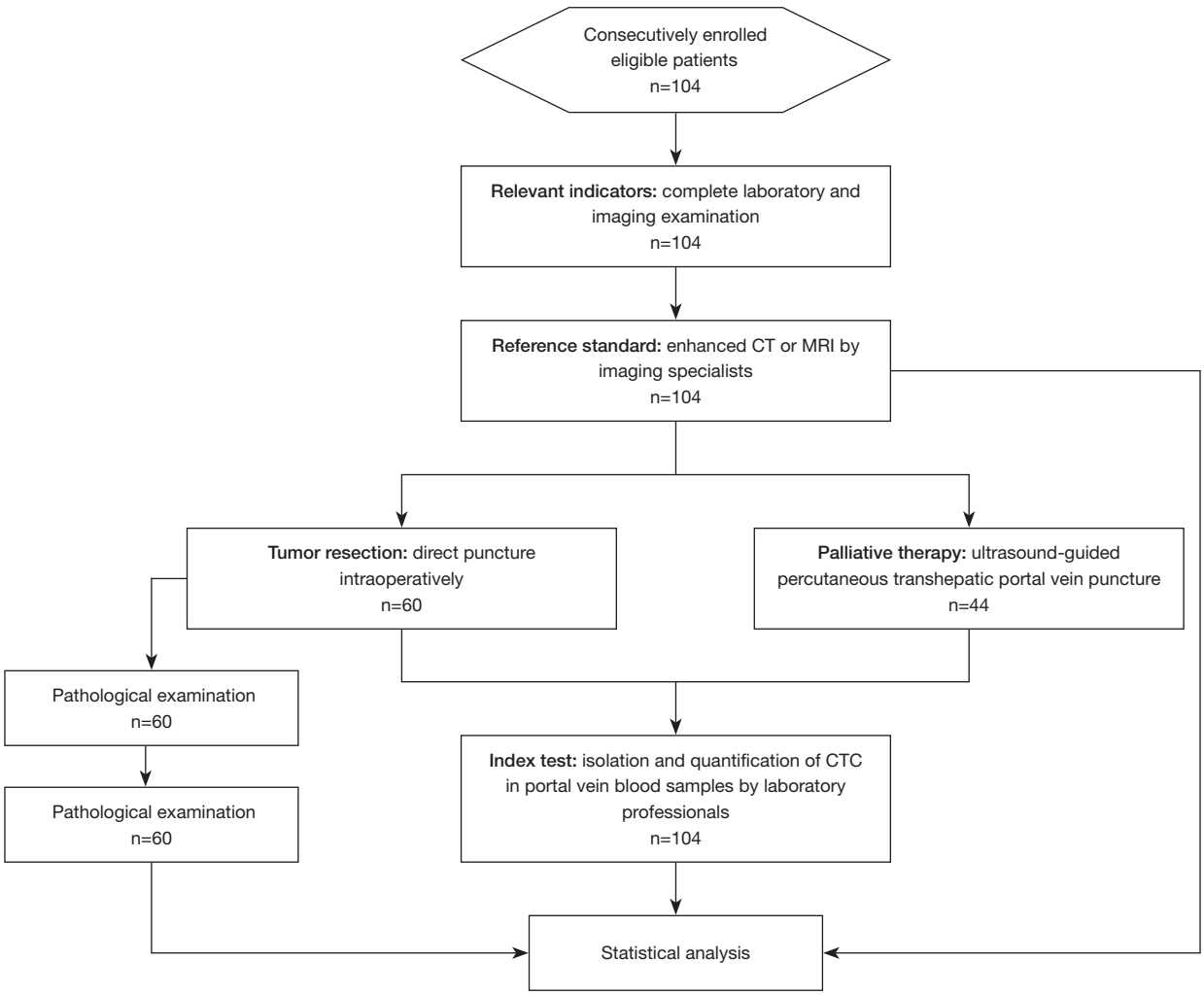

Figure S1 Research flow chart. CT, computed tomography; MRI, magnetic resonance imaging; CTC, circulating tumor cell.

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