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ORIGINAL ARTICLE

Diagnostic Value of Different Phenotype Circulating Tumor Cells in Hepatocellular Carcinoma

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Abstract

Background A growing body of research indicates that the monitoring of circulating tumor cells (CTCs) may have great significance to the diagnosis of malignant tumors, assessment of condition, selection of treatment methods, and evaluation of prognosis and has a broad range of potential applications. However, the value of CTCs with different phenotypes in the diagnosis of hepatocellular carcinoma (HCC) and assessment of patient condition remains unclear.

Methods We collected 5 ml of peripheral blood from 176 patients who were found to have space-occupying lesions in the liver via B-ultrasound diagnosis at Zhujiang Hospital affiliated with Southern Medical University between August 2015 and October 2017 and used CanPatrol™ CTCs assay technology to isolate and count CTCs with different phenotypes in the patients' peripheral blood. This allowed analysis of the value of CTCs with different phenotypes in the diagnosis of HCC and assessment of BCLC stage.

Results We used CanPatrol™ CTCs assay technology to isolate different types of CTCs: epithelial CTCs (only stained for epithelial markers), mesenchymal CTCs (only stained for mesenchymal markers), mixed CTCs (stained for epithelial markers and mesenchymal markers), and total CTCs (all of the foregoing CTC phenotypes). Of 176 observed patients, 6 patients were finally diagnosed as other malignant tumor liver metastasis, 113 were diagnosed as having hepatocellular carcinoma, and 57 were diagnosed as having nonmalignant liver diseases. Furthermore, we intend to evaluate the diagnostic value of different phenotype CTCs count in discrimination between hepatocellular carcinoma and nonmalignant liver diseases. We found that CTCs of all types were significantly more numerous in the peripheral blood of the HCC group patients than in the NLD group patients ($P < 0.05$). Furthermore, of the different types of CTCs, total CTCs had the greatest diagnostic value (AUC 0.774; 95% CI, 0.704–0.834). A further discovery was that the AUC values for total CTCs, AFP, and a combined model (combined use of total CTCs and AFP) were 0.774 (95%CI, 0.704–0.834), 0.669 (95%CI, 0.587–0.750), and 0.821 (95%CI, 0.756–0.886). Late-stage HCC patients (BCLC stage B-C) had a higher peripheral blood mesenchymal CTC count than early-stage patients (BCLC stage 0-A) (median:1 vs 0), and mesenchymal CTCs ≥ 1 was the cut-off value for the diagnosis of BCLC stage in HCC patients (sensitivity: 66.67%, specificity: 59.46%, Youden index: 0.26).

Conclusions Total CTCs are more effective than AFP in the diagnosis of HCC; combined use of total CTCs and AFP can enhance the sensitivity of HCC diagnosis.

Keywords Hepatocellular carcinoma (HCC) · Nonmalignant liver diseases (NLD) · Circulating tumor cells (CTCs) · Diagnostic value

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumor, and over 600,000 persons worldwide die from HCC each year, which makes it the third leading cause of death among tumors.[1, 2] Because HCC is highly invasive and readily undergoes metastasis, tumors tend to grow rapidly, easily infiltrate blood vessels, and spread via

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the blood.[3] Furthermore, most HCC patients have no symptoms during the early stage. In a majority of patients, the symptoms only appear during the late stage, and a definite diagnosis can be made only after imaging; at that time, the best opportunity for treatment has already been lost.[4] However, if a prompt, clear-cut diagnosis can be made, HCC patients will have an opportunity to receive radical treatment, which will greatly increase patients' survival and quality of life. Accordingly, screening for HCC is an important approach to the early discovery of HCC cases, and serum AFP is currently the main biomarker employed in HCC screening.[5] However, the use of serum AFP in HCC screening has certain limitations: first, approximately one-third of HCC patients are negative for AFP. Second, patients with such non-HCC disorders as acute viral hepatitis, active-stage cirrhosis, and testicular cancer may also have elevated AFP.[6, 7] Hence, AFP is not a precise marker because it provides low sensitivity and specificity. As a consequence, due to its low sensitivity and specificity, AFP is not a precise marker. Accordingly, in order to boost the accuracy of HCC diagnosis, finding biomarkers that can be used in conjunction with AFP to diagnose HCC is extremely important.

Over the past decade, the detection of circulating tumor cells (CTCs) in the peripheral blood of cancer patients has gained more and more attention. Peripheral blood CTC detection can serve as an ideal source for sampling because it is a simple, repeatable, and minimally invasive procedure.[8–10] CTCs refer to the tumor cells that exist outside the solid tumors. In fact, the invasive tumor cells originated from the primary tumor proliferate constantly; they become CTCs with invasion and metastasis potentials after leaving the tumor tissue and entering blood circulation.[9, 11] Recently, the function of CTCs in tumor diagnosis, recurrence, and metastasis has been under active investigation.[12–14] The value of CTCs in the diagnosis of HCC still remains relatively unknown. Accordingly, in this study, we analyzed and compared differences in the types of CTCs in the peripheral blood of HCC patients and patients with nonmalignant liver diseases as part of a preliminary investigation of the clinical value of different types of CTCs in the diagnosis of HCC and assessment of patient condition. The goals of this study were to enhance the accuracy of HCC diagnosis and effectively assess the condition of HCC patients. This cannot only conserve medical resources and reduce patients' medical usage, but also facilitate the determination of further treatment regimens.

Materials and Methods

Study Design

We took samples of peripheral blood and tested serum AFP levels when patients entering this hospital were found by B-ultrasound to have space-occupying lesions in the liver. We then used the CanPatrol™ System (Surexam Biotech, Guangzhou, China) to isolate and count CTCs with different phenotypes.[15, 16] It was confirmed that those patients had not received neoadjuvant chemotherapy or radiotherapy before enrollment. In addition, patients who presented with a diagnosis of other malignant tumor were also excluded from this study. Pathological verification was performed in the case of all HCC patients, and tumor stage was determined according to the Barcelona Clinic Liver Cancer (BCLC) staging classification.[17] Complete clinical data including history, physical examination, laboratory, and radiographic evaluations were also collected.

Detection Different Phenotype CTCs Using the CanPatrol™ System

A 5-mL sample of peripheral blood from each patient was placed in a K2-ethylenediaminetetraacetic acid (EDTA) tube and centrifuged ($600g \times 5$ min) to collect cell pellets. The supernatant was discarded, and the pellet was resuspended in 5 mL of phosphate-buffered saline (PBS). The cell suspension was then passed through a filter tube (Surexam Biotech, Guangzhou, China) containing a membrane filter (Millipore, Billerica, MA, USA) with a pore size of 8 μm under a vacuum, for collection of CTCs remaining on the filter. Blood cells are smaller than CTCs, so they readily pass through the filter.

Three sets of nucleic acid probes were used to detect and characterize the expression of epithelial and mesenchymal genes in CTCs, using multiplex RNA with in situ hybridization (RNA-ISH). The first set of probes had four epithelial transcripts (CK8, CK18, CK19, and EpCAM). The second set of probes had two mesenchymal transcripts (Vimentin and Twist), which are CD45 transcripts used to distinguish leukocytes from CTCs. Cells remaining on the filter were permeabilized and then digested with protease, followed by a series of hybridization steps using the different probes. A previous study provided details of the hybridization procedures.

Finally, DAPI was used to stain the cell nucleuses. The red and green dots of the fluorescent signal observed in the cells represented the epithelial and mesenchymal gene expression, respectively. The bright blue fluorescent dots showed that the CD45 gene expression was the marker of the white blood cells. The assays were applied in both selected HCC specimens and all blood specimens.[15, 18]

AFP Measurements

Serum AFP was measured using an ELISA Kit (Quantikine Human AFP Immunoassay Kit; R&D Systems, Minneapolis, MN, USA). The assay was performed according to manufacturer instructions.

Statistical Analysis

The data were statistically analyzed using SPSS 21.0 statistical software. CTC counts are presented as medians with interquartile ranges for each sub-type and were compared using the Mann-Whitney *U* test. Receiver operating curve (ROC) analysis was used to assess the ability to differentiate HCC from NLD based on different CTC parameters. These results are presented as area under the ROC curves (AUC), with 95% confidence intervals (95% CI) and *p* values. The diagnostic results, sensitivity, specificity, and Youden index were calculated. The cut-off point corresponding to the maximum of the Youden index was taken as the best critical point for clinical diagnosis. All the tests were bilateral, and the significance level was set at $\alpha = 0.05$.

Results

Patient Characteristics

From August 2015 to October 2017, 176 patients in which B-ultrasound diagnosis found space-occupying lesions in the liver were selected for inclusion in this study. All included patients underwent whole-body CT, PET, or pathology to confirm diagnosis. It was found that other malignant tumors had metastasized to the liver in six cases (three with lung cancer liver metastases, two with colorectal liver metastases, one with pancreatic cancer liver metastases), and these six patients were excluded. Of the remaining 170 patients, 113 were diagnosed with HCC (74 cases of BCLC stage 0-A, 39 cases of BCLC stage B-C) and 57 were diagnosed with NLD (18 hepatic cyst patients, 5 liver abscess patients, 14 liver cirrhosis nodule patients, 5 granulomatous inflammation patients, 6 hepatic hemangioma patients, and 9 nodular regenerative hyperplasia patients). As a result, a total of 113 HCC patients and 57 NLD patients were included in this study (Fig. 1a). No significant difference in any patient characteristic was found between hepatocellular carcinoma patients and nonmalignant liver disease patients.

Different Phenotype CTC Count in HCC and Nonmalignant Liver Disease Patients

We performed incubation with tumor epithelial cells and mesenchymal marker-specific antibodies and used specific immunofluorescence to perform marking, which allowed the isolation and identification of different phenotype CTCs. In fluorescence microscope observation, the epithelial marker was represented in red fluorescence, and the mesenchymal marker was represented in green fluorescence. Combining testing results, the relative expression of epithelial and mesenchymal markers allowed CTCs to be classified as three phenotypes, namely epithelial CTCs (containing only epithelial markers), mesenchymal CTCs (containing only mesenchymal markers), and mixed CTCs (containing both epithelial and mesenchymal markers) (Fig. 1b).

The differences between different phenotype CTC counts in HCC and NLD patients were further investigated. After analyzing the CTCs in the peripheral blood of the 113 HCC patients and 57 NLD patients, we determined the epithelial CTCs (median:0vs0), mixed CTCs (median:2vs0), mesenchymal CTCs (median:0vs0), and total CTCs (total CTCs of all phenotypes) (median:4vs1) in the HCC and NLD groups. Sequencing and testing revealed that levels of CTCs of all phenotypes in the peripheral blood of HCC group patients were significantly higher than in the case of NLD group patients ($P < 0.05$) (Table 1). The CTC cut-off value in each type of HCC diagnosis was further determined: total CTCs ≥ 3 indicated positive (sensitivity: 61.95%, specificity: 89.47%,Youden index: 0.51), epithelial CTCs ≥ 1 indicated positive (sensitivity: 45.13%, specificity: 78.95%, Youden

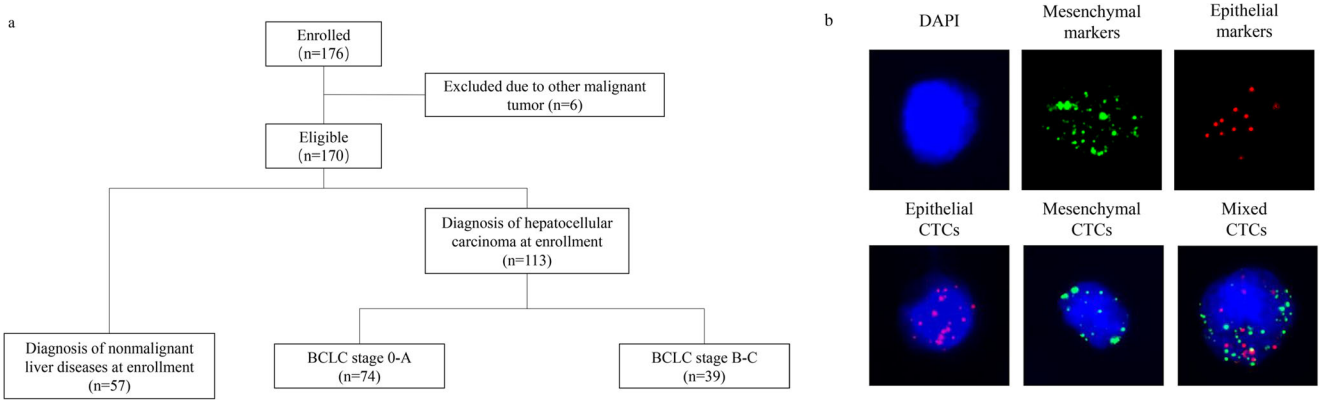

Fig. 1 **a** Flowchart of diagnosis of patients enrolled in the study. **b** Examples of different phenotype CTCs under automated imaging fluorescent microscope: epithelial CTCs stained for epithelial markers (red dots); mesenchymal CTCs stained for mesenchymal markers (green dots); mixed CTCs stained for epithelial markers (red dots) and mesenchymal markers (green dots)

Table 1 The comparison of different phenotype CTC count between HCC and NLD (median and interquartile range)

	Total CTCs	Epithelial CTCs	Mixed CTCs	Mesenchymal CTCs
HCC ($n = 113$)	4 (1, 10)	0 (0, 2)	2 (0, 6)	0 (0, 2)
NLD ($n = 57$)	1 (0, 2)	0 (0, 0)	0 (0, 1)	0 (0, 0)
Z	− 5.903	− 3.383	− 4.317	− 4.825
P value	< 0.001	0.001	< 0.001	< 0.001

$P < 0.05$ was considered statistically significant

index: 0.24), mixed CTCs ≥ 2 indicated positive (sensitivity: 53.10%, specificity: 82.46%, Youden index: 0.36), and mesenchymal CTCs ≥ 1 indicated positive (sensitivity: 49.56%, specificity: 87.72%, Youden index: 0.37). Among these types, total CTCs had the largest AUC (0.774; 95% CI, 0.704–0.834). In comparison, the diagnostic efficiency of the traditional biomarker AFP was 0.669 (sensitivity: 44.25%, specificity: 89.47%) (Table 2). Accordingly, in the efficiency of the different phenotype CTCs and AFP in the diagnosis of HCC, total CTCs had the greatest diagnostic value (AUC: 0.774, sensitivity: 61.95%, specificity: 89.47%).

The Combination of Total CTCs and AFP in the HCC Diagnosis

In order to enhance the accuracy of diagnosis, we assessed the effectiveness of the use of AFP alone or total CTCs, or a combined model (combining total CTCs and AFP) in the diagnosis of HCC. Our results indicated that the AUC values for total CTCs, AFP, and the combined model were 0.774 (95%CI, 0.704–0.834), 0.669 (95%CI, 0.587–0.750), and 0.821 (95%CI, 0.756–0.886) (Fig. 2). Based on these evidence, total CTCs have superior diagnostic value to AFP, and the combined use of total CTCs and AFP can boost diagnostic effectiveness compared with the use of total CTCs or AFP alone.

Mesenchymal CTC Test for Prediction of BCLC Stage

Among the 113 HCC patients, differences in different phenotype CTCs had no correlation with age and sex. The mesenchymal CTCs of the peripheral blood of late-stage HCC patients (BCLC stage B-C) were higher than that of early-stage patients (BCLC stage 0-A) (median:1 vs 0), and the difference had statistical significance (Fig. 3). We further determined that mesenchymal CTCs ≥ 1 could be employed as a cut-off value for the determination of the BCLC stage of HCC patients (sensitivity: 66.67%, specificity: 59.46%, Youden index: 0.26) (Table 3).

Discussion

In the previous study, we employed CanPatrol™ CTC assay technology to assess the value of different phenotype CTCs in the diagnosis of HCC, and it has already been verified that mesenchymal CTCs are independent risk factors for recurrence of HCC[16]. Many types of CTC assay methods are currently in use; of them, the CellSearch system is currently in the broadest use. This technology combines anti-EpCAM antibody magnetic beads with tumor cell antigens to create antigen-antibody-magnetic bead immune complexes, which are used to achieve the goal of isolating and enriching CTCs.[19, 20] However, because EpCAM is limited to CTCs with epithelial sources, and a growing number of studies indicate that epithelial CTCs can undergo a epithelial-to-mesenchymal transition (EMT), which causes the expression

Table 2 The diagnostic efficiency of different phenotype CTCs in differentiating patients with HCC and NLD

	Cutoff	Sensitivity	Specificity	*P*	Youden index	AUC (95% CI)
AFP	400 μg/L	44.25%	89.47%	< 0.001	0.34	0.669 (0.587–0.750)
Total CTCs				< 0.001		0.774 (0.704–0.834)
	≥ 2	72.57%	61.40%		0.34	
	≥ 3	61.95%	89.47%		0.51	
	≥ 4	50.44%	92.98%		0.43	
Epithelial CTCs				0.003		0.637 (0.554–0.721)
	≥ 1	45.13%	78.95%		0.24	
	≥ 2	28.32%	91.23%		0.20	
Mixed CTCs				< 0.001		0.696 (0.619–0.772)
	≥ 1	66.37%	50.88%		0.17	
	≥ 2	53.10%	82.46%		0.36	
Mesenchymal CTCs				< 0.001		0.696 (0.618–0.774)
	≥ 1	49.56%	87.72%		0.37	
	≥ 2	30.44%	92.98%		0.23	

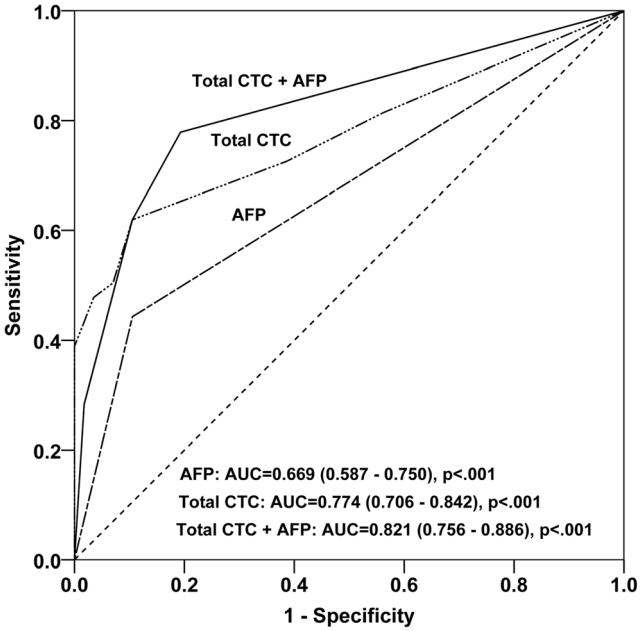

Fig. 2 ROC curve showing the effectiveness of the use of AFP alone, total CTCs alone, and the combined use of total CTCs and AFP in the diagnosis of HCC

of epithelial markers including EpCAM to be down-regulated or even absent, and the expression of mesenchymal markers to be up-regulated and obtain mesenchymal phenotypes, forming CTCs with different phenotypes. This limits the ability of the CellSearch to detect CTCs.[21] Furthermore, the morphology, cytoskeleton, and biological characteristics of tumor cells may change after they undergo EMT, giving the tumor cells even greater infiltration, migration, and in vivo survival abilities.[22] Accordingly, the use of epithelial markers alone to isolate and identify CTCs can only detect those CTCs with epithelial markers and may not catch mesenchymal CTCs, which are more closely connected with tumor progression.[23] Furthermore, the presence of EMT-related markers on CTCs has been reported to more accurately predict tumor progression than the expression of epithelial markers alone.[24] In the patients with breast cancer, the ratio of mesenchymal CTCs and epithelial CTCs (M+/E+) was increased while breast cancer progression whereas the M+/E+ ratio was decreased while breast cancer was reduced.[25] Sun T et al. found that the up-regulation of Twist1 (an HCC mesenchymal marker) increased the invasiveness and mobility of tumor cells and promotes tumor angiogenesis, which leads to the HCC progression.[26] In addition, the latest researches indicated that mesenchymal CTCs were highly associated with tumor metastasis and prognosis such as HCC, colorectal cancer, and lung cancer.[27–29] Nevertheless, the use of CTCs still faces many hurdles because the quantity of CTCs in peripheral blood is extremely low and the heterogeneity of CTCs is wide. According to the previous findings, the low quantity of CTCs is due to that the most of CTCs are eliminated by host immune system or anoikis while they were entering the peripheral circulation. Only a few CTCs with highly invasiveness and metastasizing were able to survive in the peripheral circulatiing system.[30, 31] Moreover, the survival CTCs may be shed from different locations within tumors, which are heterogeneous in nature, and even from metastases. And there are also differences in the survival, invasion, and metastasis of different phenotype of CTCs in peripheral blood.[9] These characters of the survival CTCs reveal the importance and unique for the diagnosis of malignant tumors, assessment of patients' condition, effectiveness of short-/long-term treatment, and prognosis monitoring, as well as the other potential applications.[32]

To date, however, the effectiveness of the use of EMT-related phenotype CTCs in diagnosis of HCC remains unclear. This study therefore used CanPatrol™ assay technology[15, 16] and classified CTCs as epithelial CTCs (containing only epithelial markers), mesenchymal CTCs (containing only mesenchymal markers), and mixed CTCs (simultaneously containing epithelial and mesenchymal markers) on the basis of the expression of epithelial markers (CK8, CK18, CK19, and EpCAM) and mesenchymal markers (Vimentin and Twist) (Fig. 1b). This approach enabled the isolation and identification of different phenotype CTCs, and the investigation of their value in the diagnosis of HCC and assessment of patient condition.

Early diagnosis and treatment are key to achieving improved clinical outcomes in HCC patients. AFP is an alpha-globulin and is the most widely used biomarker in HCC diagnosis.[6, 7] Although serum AFP possesses high specificity in the diagnosis of HCC, AFP levels are not elevated in some early-stage HCC patients and may become elevated only after patients reach a later stage. In addition, AFP levels may display no abnormalities at any stage in some HCC patients.[33, 34] As a consequence, it may be impossible to obtain a prompt diagnosis in the case of some HCC patients, which will cause the optimal treatment period to be missed. Accordingly, reliance on serum AFP levels alone to diagnose HCC has certain limitations. However, even during early-stage HCC, some tumor cells may leave the tumor foci and entered the peripheral blood, where they take the form of CTCs, and CTCs can consequently serve as optimal biomarkers in the diagnosis of HCC.[9, 35]

In our study, we found that levels of CTCs with all phenotypes were higher in the peripheral blood of patients in the HCC group than in patients in the NLD group ($P < 0.05$, Table 1). Further analysis revealed that total CTCs have the greatest diagnostic value (AUC: 0.774; 95% CI, 0.704–0.834), with an optimal HCC diagnosis cut-off value of 3 (patients with 3 or more CTCs are judged to be positive). After also assessing the effectiveness of the conventional biomarker AFP and a combined model (combining the use of total CTCs and AFP) in the diagnosis of HCC, we found that the diagnostic effectiveness of these approaches was combined model (AUC: 0.821, 95%CI, 0.756–0.886) > total CTCs (AUC: 0.774, 95%CI, 0.704–0.834) > AFP (AUC: 0.669, 95%CI, 0.587–0.750) (Table 2, Fig. 2). It can be

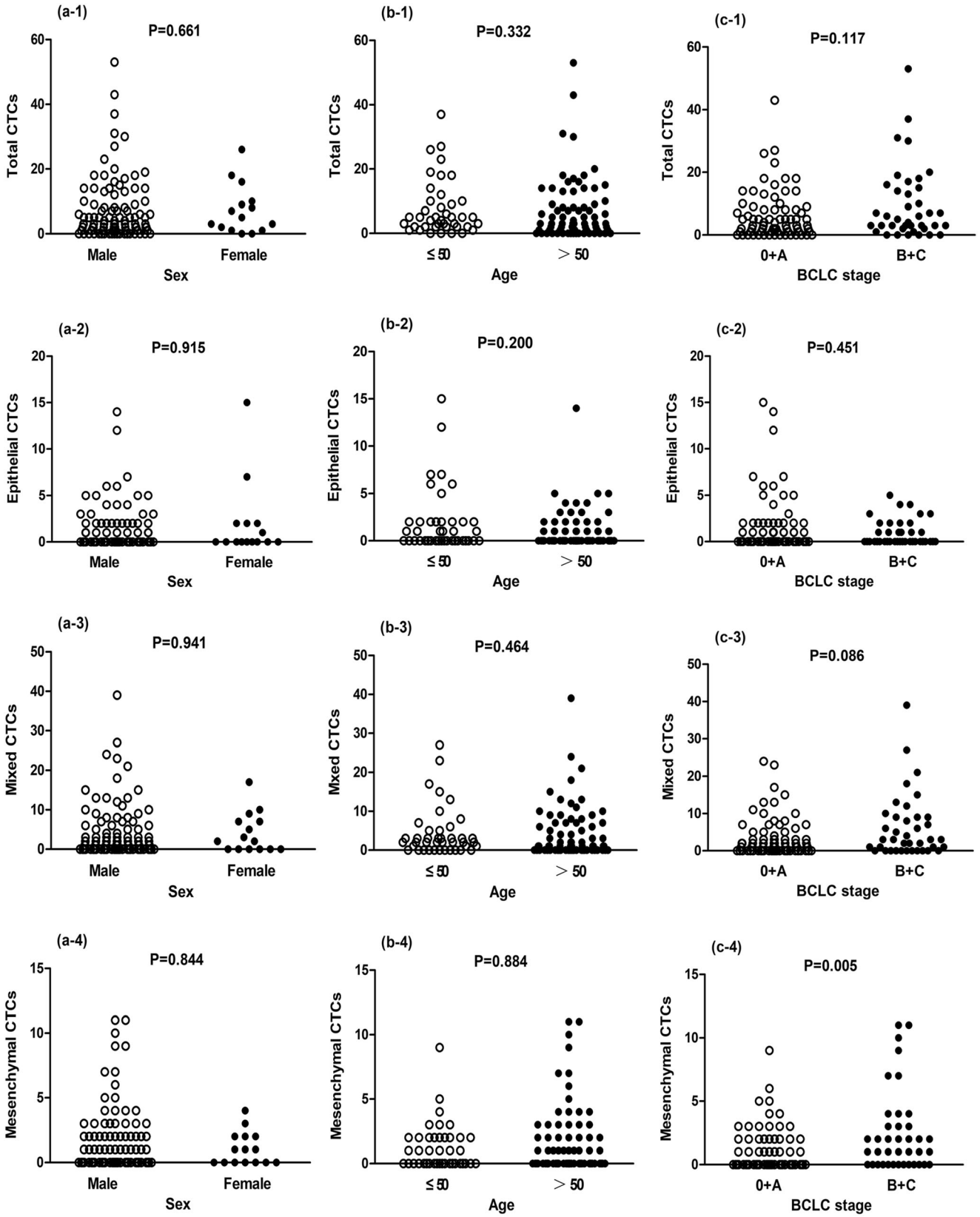

Fig. 3 Distribution of different phenotype CTC count in HCC patients according to sex, age, and BCLC stage. **a** Sex, **b** age, and **c** BCLC stage

seen from this that total CTCs were more effective than AFP in the diagnosis of HCC, and the combination of total CTCs and AFP can enhance diagnostic effectiveness. Accumulating findings indicated that the combination of AFP and the other

Table 3 The diagnostic efficiency of mesenchymal CTCs in differentiating HCC patients with BCLC stage 0-A and BCLC stage B-C

Cut-off	Sensitivity	Specificity	Youden index
Mesenchymal CTCs			
≥1	66.67%	59.46%	0.26
≥2	48.72%	71.62%	0.20
≥3	30.77%	83.78%	0.15

biomarkers significantly increases the effectiveness of HCC diagnosis.[36, 37]Therefore, we believe that multiple biomarkers would be feasible for HCC diagnosis which could remedy the sensitivity and specificity of conventional single biomarker diagnosis.

In order to further analyze the diagnostic value of different phenotype CTCs in the assessment of HCC status, we compared the CTC count of peripheral blood from late-stage (BCLC stage B-C) and early-stage HCC patients (BCLC stage 0-A). We found that among the different CTC phenotypes, only mesenchymal CTC count was significantly higher in late-stage patients than in early-stage patients (median: vs), which allowed us to confirm that mesenchymal CTCs ≥ 1 can serve as a cut-off value in the determination of the BCLC stage of HCC patients (sensitivity: 66.67%, specificity: 59.46%, Youden index: 0.26) ($P < 0.0$, Fig. 3, Table 3). Recent research has indicated that EMT-related marker expression in CTCs provides important information for the evaluation of clinical outcomes. The ratio of epithelial/mesenchymal CTCs markers in the peripheral blood of tumor patients changes with treatment and prognosis, and when treatment achieves remission, the ratio of epithelial/mesenchymal markers will increase significantly. However, when the disease progresses, and the patient's condition worsens, the expression of epithelial/mesenchymal markers will be down-regulated.[33, 38] Our results indicate that mesenchymal CTCs have important significance in the assessment of HCC patients' conditions. BCLC stage cannot only be used to assess HCC patients' condition and prognosis, but is also an important marker that can be used in determining whether patients can receive radical resection.[39] Therefore, mesenchymal CTCs in patients' peripheral blood may therefore serve as biomarkers that can be used to assess HCC patients' conditions and assist selection of treatment methods. Regardless of the unique characters of the CTC evaluation, it remains an indirect method for the assessment of HCC patients' condition, so the tumor tissue biopsies are still the "Gold Standard" for assessment of HCC patients' condition and prognosis. Therefore, we will focus on the comparison of the effectiveness of conventional tissue biopsies and different phenotype CTCs in the assessment of HCC condition in the subsequent investigations. Furthermore, we will explore the value of different phenotype CTCs in the evaluation of HCC patients' condition as well. In addition, the limitations of this study are its relatively small cohort size and data from a single study center. A prospective, multicenter, randomized clinical trial should be designed to further validate the diagnostic value of different phenotype CTCs in HCC.

Conclusions

In conclusion, we found that different phenotype CTCs may all serve as biomarkers in the diagnosis of HCC. Among the types, total CTCs were most effective in diagnosing HCC, and the use of total CTCs and AFP in combination can boost the effectiveness of HCC diagnosis. In addition, mesenchymal CTCs can be used as biomarkers in determining tumor stage in HCC patients. In summary, this study provided a scientific basis for the use of testing of different phenotype CTCs in patients' peripheral blood as a means of diagnosing HCC.

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Availability of Data and Materials Please contact author for data requests.

Authors' Contributions Yuan Cheng, Lei Luo, and Juqiang Zhang contributed equally to this work and should be considered as co-first authors. Yuan Cheng, Lei Luo, Juqiang Zhang, and Mantian Zhou performed the experiments. Yujun Tang, Guolin He, and Yishi Lu collected patients' information. Zhong Wang and MingXin Pan designed the study, edited the manuscript, and confirmed the data presented in the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate This study protocol was approved by the Institutional Review Board of the Second Affiliated Hospital of Southern Medical University (ZJYY-2015-GDEK-001). All participants will provide informed written consent prior to their entry into the study. In the case that changes in the protocol are necessary, relevant amendments will be made and submitted to the ethics trial registration authorities for approval.

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