Circulating tumor cells with stem-like phenotypes for diagnosis, prognosis and therapeutic response evaluation in hepatocellular carcinoma

Wei Guo*, Yun-Fan Sun*, Min-Na Shen*, Xiao-Lu Ma, Jiong Wu, Chun-Yan Zhang, Yan Zhou, Yang Xu, Bo Hu, Min Zhang, Gang Wang, Wei-Qin Chen, Lin Guo, Ren-Quan Lu, Chao-Hui Zhou, Xin Zhang, Ying-Hong Shi, Shuang-jian Qiu, Bai-shen Pan, Ya Cao, Jian Zhou, Xin-Rong Yang and Jia Fan.

Authors and affiliations:

1Department of Laboratory Medicine, Zhongshan Hospital, Fudan University, Shanghai, P. R. China (Wei Guo, Min-Na Shen, Xiao-Lu Ma, Jiong Wu, Chun-Yan Zhang, Yan Zhou, Bai-shen Pan)

2Department of Liver Surgery, Liver Cancer Institute, Zhongshan Hospital, Fudan University; Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Shanghai, P. R. China (Xin-Rong Yang, Yun-Fan Sun, Yang Xu, Bo Hu, Xin Zhang, Ying-Hong Shi, Shuang-jian Qiu, Jian Zhou, Jia Fan).

3Institute of Biomedical Sciences, Fudan University, Shanghai, P. R. China (Jian Zhou, Jia Fan).

4Department of Clinical Laboratory, Shanghai Public Health Clinical Center,
Fudan University, Shanghai, P. R. China (Min Zhang, Gang Wang).

5 Department of Clinical Laboratory, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, P. R. China (Wei-Qin Chen;)

6 Department of Laboratory Medicine, Shanghai Cancer Center, Fudan University, Shanghai, P. R. China (Lin Guo, Ren-Quan Lu).

7 Department of Gastroenterology, Zhongshan Hospital, Fudan University, Shanghai, P. R. China (Chao-Hui Zhou);

8 Cancer Research Institute, Xiangya School of Medicine, Central South University; Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Changsha 410078, China (Ya Cao).

*These authors contributed equally to this work.

Corresponding author

Jia Fan, MD, PhD, Liver Cancer Institute, Fudan University, 136 Yi Xue Yuan Road, Shanghai 200032, P. R. China. Tel. & Fax: +86-21-64037181. E-mail: fan.jia@zs-hospital.sh.cn

Or to

Xin-Rong Yang, MD, PhD, Liver Cancer Institute, Fudan University, 136 Yi Xue Yuan Road, Shanghai 200032, P. R. China. Tel. & Fax: +86-21-64037181. E-mail: yang.xinrong@zs-hospital.sh.cn
Running title

CTC with stem-like phenotype for HCC diagnosis and prognosis

Keywords

hepatocellular carcinoma, circulating tumor cells, diagnosis, prognosis, therapeutic response evaluation.

Abbreviations: HCC, hepatocellular carcinoma; LC, liver cirrhosis; CHB, chronic hepatitis B infection; BHL, benign hepatic lesion; HD, healthy donor; CTC, circulating tumor cell; AFP, α-fetoprotein; HBV, hepatitis B virus; ROC, receiver operating characteristics; TTR, time to recurrence

Declaration of interest:

We declare that we have no conflicts of interest.

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Author contributions:

Drs. Wei Guo and Jia Fan had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data.
analysis.

Study concept and design: Wei Guo, Xin-Rong Yang, Jia Fan

Acquisition of data: Xin-Rong Yang, Yun-Fan Sun, Min Zhang, Gang Wang, Wei-Qin Chen, Lin Guo, Ren-Quan Lu, Chao-Hui Zhou, Min-Na Shen, Xiao-Lu Ma, Jiong Wu, Chun-Yan Zhang, Yan Zhou, Bo Hu, Xin Zhang, Ya Cao

Analysis and interpretation of data: Wei Guo, Xin-Rong Yang, Yun-Fan Sun

Drafting the manuscript: Wei Guo, Xin-Rong Yang, Yun-Fan Sun, Xiao-Lu Ma

Critical revision of the manuscript for import intellectual content: Xin-Rong Yang, Yun-Fan Sun, Yang Xu, Ying-Hong Shi, Jia Fan, Bai-shen Pan, Shuang-jian Qiu

Statistical analysis: Wei Guo, Xiao-Lu Ma, Jiong Wu

Obtained funding: Jia Fan

Administrative, technical, and material support: Jian Zhou, Jia Fan

Study supervision: Jia Fan

Email Addresses of Authors:

Wei Guo, guo.wei@zs-hospital.sh.cn

Yun-Fan Sun, yunfan_sun@msn.com

Min-Na Shen, shen.minna@zs-hospital.sh.cn

Xiao-Lu Ma, dadagis13@163.com

Jiong Wu, wu.Jiong@zs-hospital.sh.cn

Chun-Yan Zhang, zhang.chunyan@zs-hospital.sh.cn
Yan Zhou, zhou.yan3@zs-hospital.sh.cn

Yang Xu, xu.yang@zs-hospital.sh.cn

Bo Hu, bohu1120@hotmail.com

Min Zhang, Zhangminlisa@126.com

Gang Wang, 37729685@qq.com

Wei-Qin Chen, wonderforjune@126.com

Lin Guo, Guolin500@hotmail.com

Ren-Quan Lu, lurenquan@126.com

Chao-hui Zhou, Zhou.chaohui@zs-hospital.sh.cn

Xin Zhang, Zx64182754@hotmail.com

Ying-Hong Shi, shi.yinghong@zs-hospital.sh.cn

Shuang-Jian Qiu, qiu.shuangjian@zs-hospital.sh.cn

Bai-shen Pan, pan.baishen@zs-hospital.sh.cn

Ya Cao, ycao98@vip.sina.com

Jian Zhou, zhou.Jian@zs-hospital.sh.cn

Xin-Rong Yang, yang.xinrong@zs-hospital.sh.cn
**Translational Relevance**

Whether detection of circulating tumor cells (CTCs) with stem-like phenotypes could be a reliable and effective method that integrates in HCC early diagnosis, outcome prediction as well as treatment response evaluation was unknown. In this study, we constructed a CTC panel including four putative stem cell biomarkers (epithelial cell adhesion molecule, CD133, CD90, and cytokeratin 19). In our clinical trial including 1006 individuals, the AUC of this panel is 0.88 (sensitivity=72.5%, specificity=95.0%, PPV=92.4, NPV=77.8) in training set and 0.93 (sensitivity=82.1%; specificity=94.2%, PPV=89.9, NPV=89.3) in validation set. HCC patients with high CTC load show higher recurrence rate than patients with low load (49.1% vs. 24.7%). Our CTC panel showed high sensitivity and specificity in the diagnosis of HCC and could be a real-time parameter for risk prediction and treatment monitoring in treatment response surveillance.
Abstract

Background

In present study, we assessed the clinical value of circulating tumor cells (CTCs) with stem-like phenotypes for diagnosis, prognosis and surveillance in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) by an optimized QPCR-based detection platform.

Methods

Differing subsets of CTCs were investigated, and a multimarker diagnostic CTC panel was constructed in a multicenter-patient study with independent validation (total n=1006), including healthy individuals, patients with chronic hepatitis B infection (CHB), liver cirrhosis (LC), benign hepatic lesion (BHL) and HBV-related HCC, with area under the receiver operating characteristic curve (AUC-ROC) reflecting diagnostic accuracy. The role of CTC panel in treatment response surveillance and its prognostic significance were further investigated.

Results

The AUC of CTC panel was 0.88 in training set [sensitivity=72.5%, specificity=95.0%, positive predictive value (PPV)=92.4, negative predictive value (NPV)=77.8] and 0.93 in validation set (sensitivity=82.1%; specificity=94.2%, PPV=89.9, NPV=89.3). This panel performed equally well in detecting early-stage and \( \alpha \)-fetoprotein (AFP)-negative HCC, as well as differentiating HCC from CHB, LC and BHL. The CTC load was decreased
significantly after tumor resection, and patients with persistently high CTC load showed a propensity of tumor recurrence after surgery. The prognostic significance of CTC panel in predicting tumor recurrence was further confirmed (training: HR=2.692, 95% CI, 1.617-4.483, \( P<0.001 \); validation: HR=3.127, 95% CI, 1.360-7.190, \( P=0.007 \))

Conclusions

Our CTC panel showed high sensitivity and specificity in HCC diagnosis and could be a real-time parameter for risk prediction and treatment monitoring, enabling early decision-making to tailor effective antitumor strategies.
Introduction

Hepatocellular carcinoma (HCC) is the most prevalent malignancy in the world and ranks the second as the cause of cancer deaths.\(^1\) For the absence of an effective method for timely diagnosis, only 30-40% of HCC patients qualifying for potentially curative treatments at the time of diagnosis.\(^2\) Meanwhile, even after curative treatment, approximate 60%-70% patients experience recurrence or distant metastasis within 5 years.\(^3\) Although serologic tumor markers, clinicopathologic parameters, and radiologic modalities are commonly used in routine clinical practice for management of HCC patients,\(^4\) none of these approaches can provide comprehensive and precise information covering diagnosis, outcome prediction, and the evaluation of therapeutic response in HCC.\(^5\) The lack of a reliable and versatile method that integrates early diagnosis, precisely prediction and real-time surveillance become the main obstacle for further improving the clinical outcome of HCC patients.

Ready and noninvasive access to circulating tumor cells (CTCs) is advantageous, constituting a potential surrogate for tumor biopsy with new diagnostic, prognostic, and therapeutic import.\(^6\)\(^-\)\(^8\) However, evidence indicates that among thousands of cells freeding into circulation from primary tumors, only small populations with stem cell-like properties have the generative capacity for driving tumor progression, metastasis and resistance to traditional therapies.\(^9\)\(^,\)\(^10\) Thus, identifying the subpopulations of CTC with stem cell phenotypes might be more clinically relevant than the analyses on total CTC
counts. Recently, we identified the stem cell features of epithelial cell adhesion molecule (EpCAM) CTCs and their clinical significance in HCC. Others have also characterized other subpopulations of CTC with stem-like cell features in HCC, based on various surface molecules, such as CD44, CD90, and intercellular adhesion molecule 1 (ICAM1). These observations imply that CTCs with stem-like cell features in HCC are phenotypically diverse, which is in agreement with our previous study that acknowledged not only the heterogeneity of cancer stem cell (CSC) markers expression in HCC, but also the array of CSC subsets that exists and that may vary from patient to patient. Therefore, targeting of CTCs with stem-like phenotypes through multimarker strategies might be optional choice for CTCs detection in HCC patients.

Our previous study constructed a novel optimized platform for CTC detection in HCC patients, based on quantitative real-time polymerase chain reaction (qRT-PCR) analysis and negative enrichment strategy. Based on this CTC detection platform, we further systematically screened the expression patterns of nine putative CSC biomarkers (EpCAM, CD90, CD24, ATP-binding cassette subfamily G member 2 (ABCG2), CD44, Nestin, CD133, cytokeratin19 (CK19), and ICAM1) in CTCs of HCC patients, and a multimarker diagnostic panel, targeting of CTCs subpopulation with stem-like phenotypes, was thereby constructed through a multicenter patient study with independent validation, via a large group of 1006 subjects including healthy...
individuals, patients with chronic hepatitis B infection (CHB), liver cirrhosis (LC), benign hepatic lesion (BHL) and hepatitis B virus (HBV)-related HCC. Moreover, the treatment response surveillance and the prognostic significance of our CTC panel were further investigated in the same HCC group. We found the CTC detection panel showed considerable clinical benefit in HCC early diagnosis, outcome prediction as well as treatment response evaluation.
Methods and Materials

Study design and patient selection
We recruited consecutive patients with HCC, CHB, LC, BHL and healthy donor (HD) from four clinical institutions in Shanghai, China (Zhongshan Hospital, Fudan University; Shanghai Public Health Clinical Center; Shanghai Cancer Center, Fudan University; and Longhua Hospital, Shanghai University of Traditional Chinese Medicine), between December 2012, and June 2015. Blood samples were analyzed in three chronologic phases (Figure 1). First, 100 samples (HCC group, n=50; HD group, n=50) were screened for 9 putative CSC biomarkers (EpCAM, CD133, CD90, CK19, ABCG2, CD44, ICAM1, CD24, and Nestin) by qRT-PCR. Next, a multimarker diagnostic panel designed to differentiate HCC and other control groups of training group (n=401) was subjected to logistic regression and the diagnostic panel performance was validated in another independent group (n=505). For the validation group, additional BHL group (62 hepatic hemangioma, 26 hepatic cyst, 10 focal nodular hyperplasia, and 2 hepatocellular adenoma) was included for further exporting the differential potential of the CTC panel. Finally, the clinical significance of CTC panel for treatment response surveillance was further explored in 60 resectable HCC patients with CTC value after resection, and the prognostic significance of the CTC panel was investigated in 195 resectable HCC patients of training set and then validated in 130 patients of validation set.
For patients received Transcatheter arterial chemoembolization (TACE), the HCC was defined according to American Association for the Study of Liver Diseases guidelines. And Serum α-fetoprotein (AFP), a serum marker routinely used for HCC surveillance in China, was assessed for adjunctive diagnosis. For resectable patients, the diagnosis of HCC was confirmed by the histologic examination of the resected primary tumor based on World Health Organization criteria. For staging, Barcelona Clinic Liver Cancer (BCLC) criteria were applied. Diagnosis of CHB required >6 months of hepatitis B surface antigen (HBsAg), HBsAg positivity, HBV DNA level >10^3 copies/ml, and elevated serum concentrations of alanine aminotransferase, according to guidelines for prevention and treatment of chronic HBV infection. Diagnosis of cirrhosis stemmed from liver biopsy and clinical imaging evidence. Diagnosis of benign hepatic lesion was based on the standard clinical, biochemical, and clinical imaging evidences, as well as the pathological data. Healthy donor subjects were eligible blood donors with normal biochemistries such as HBsAg and no liver disease (historically), viral hepatitis, or malignancies. Patients with histories of other solid tumors were excluded. The study was approved by the ethics review committee of each institution. Informed consent was obtained from participants in accordance with respective committee regulations.

Follow up for tumor recurrence
Patients treated with resectable operation were followed up every 2 months during the first postoperative year, and every 6 months afterward. Patients were monitored by serum AFP, abdomen ultrasonography, and chest X-ray every 6 months, according to the postoperative time. For patients with test suspected with recurrence, computed tomography and/or magnetic resonance imaging were used to verify whether intrahepatic recurrence and/or distal metastasis had occurred. The diagnosis of recurrence was based on typical imaging appearance in computed tomography and/or magnetic resonance imaging scan and an elevated AFP level. Follow up was began form Jan 2013 and finished on June 2015. The median follow up time was 25.2 months in training set and 21.2 months in validation set. All 325 resectable HCC patients (n=195 in training cohort and n=130 in validation cohort) have complete follow-up information.

**Blood sampling and quantitative real-time PCR**

Peripheral blood (PB) samples (5 mL each) were collected separately in different centers before initial diagnosis or one week after tumor resection and immediately transported to laboratory areas. The diagnosis, clinical information, including disease status, of each sample kept masked in the whole process of measurement until the results enter into statistical process. A qRT-PCR test platform was utilized, with negative enrichment to optimize CTC detection. In brief, samples first were negatively enriched, using RosetteSep
Human CD45 Depletion Cocktail (STEMCELL Technologies, Vancouver, BC, Canada) to minimize background expression by removing leukocyte contamination. Thereafter, RNA extraction and cDNA synthesis were performed according to manufacturer’s instructions (RNeasy® Mini Kit &QuantiTect® Reverse Transcription Kit, Qiagen, Germany), mRNA expression levels of 10 target genes (β-actin used as an internal control) were assayed by TaqMan-based real-time PCR, using a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland). All qRT-PCR assay results entailed relative quantification via $2^{-ΔΔCq}$ algorithm, where fold expression relative to a calibrator (i.e., average ΔCq of HD subjects in each group) was given. All testing was done in triplicate. Primer and probe utilization are itemized in TableS1. To calculate relative expression levels of each marker, a $2^{-ΔΔCq}$ algorithm was applied as follows, using average ΔCq of HD subjects as a calibrator: $ΔCq=Cq(\text{target})-Cq(β\text{-actin})$, with $ΔΔCq=ΔCq(\text{target})-ΔCq(\text{calibrator})$.

**Statistical Analysis**

Statistical analysis relied on standard softwares (MedCalc v10.4.7.0, MedCalc Software, Belgium; SPSS v19.0, SPSS Inc, USA and PASS v11.0.7, NCSS Inc, USA). The Mann-Whitney U test (continuous variables and non-parametric analysis) was used to test differences between groups. Backward logistic regression model was used to construct the diagnostic CTC panel with four
CSC biomarkers based on the training dataset, and variable with a $P$ value less than 0.05 was used for further model construction. Afterwards, predicted probabilities of individual study enrollees were calculated as continuous variances and functioned as surrogate markers for constructing receiver operating characteristic (ROC) curves, where area under the ROC curve (AUC) reflects diagnostic performance. The cutoff value of our CTC panel used in prognosis was estimated using X-tile 3.6.1 software (Yale University, New Haven, CT). Time to recurrence (TTR) were defined as interval between the surgery and recurrence. The cumulative recurrence and survival rates were calculated using the Kaplan-Meier method and analyzed by the log-rank test. Univariate and multivariate analyses were calculated by the Cox proportional hazards regression model. All $P$ values were two-sided, with $P<0.05$ considered statistically significant.

Based on the results obtained from the first 100 subjects for 9 CSC markers screening, a required minimum sample size of 127 patients and 77 controls was calculated by PASS with sensitivity of 0.70, specificity of 0.85 and permissible error of 0.08.
Results

Patient characteristics

The clinical characteristics of patients recruited in this study were summarized in Table 1. Groups were well-matched for serum AFP level, age, and gender (all \( P > 0.05 \), Table 1). The validation group had a significantly larger percentage of patients with multiple tumors (17.95% vs. 10.50%, \( P = 0.03 \)), larger sized tumors (52.82% vs. 40.00%, \( P = 0.02 \)), and advanced BCLC staging (51.79% vs. 34.50%, \( P < 0.01 \), Table 1). All HBV-related characteristics were well-balanced (all \( P > 0.05 \), Table 1).

Differential expression of CSC biomarkers in CTC for HCC patients

Nine candidate genes as putative CSC biomarkers were assessed by qRT-PCR in an independent and divided screening group of 100 subjects (HCC, 50; HD, 50). Levels of EpCAM, CD133, CD90, and CK19 expression were significantly higher in the HCC (vs. HD) group (Figure S1A-D), whereas ABCG2 (\( P = 0.84 \)), CD24 (\( P = 0.19 \)), CD44 (\( P = 0.27 \)), and ICAM-1 (\( P = 0.11 \)) expression levels did not differ significantly, and Nestin was uniformly undetectable (Figure S1E-I). Thus, EpCAM, CD90, CD133, and CK19 were further evaluated.

To confirm the detection accuracy of qRT-PCR method, the existence of CTC subpopulations with stem-like phenotypes was further validated by immunofluorescent method in 10 HCC patients, which were positive for four
CSC biomarkers. We found that the corresponding subpopulations of CTC were identified and well matched with the qRT-PCR detection results in HCC patients (Figure S2A). Furthermore, the single-cell transcriptional analysis also confirmed their tumor origin, such as high expression level of tumor specific gene (AFP), high expression stem cell markers (NANOG, ABCG2, SOX2), and low expression of hematopoietic markers (CD45, CD16, CD34) distinguished with leukocytes (Figure S2B).

Formulating CTC detection panel with CSC mutimarkers in training set

Expression levels of EpCAM, CD90, CD133, and CK19 were determined in a training group of 401 subjects (HCC, 200; CHB/LC, 101; HD, 100) via qRT-PCR. Relative expression levels for all four markers were significantly higher in the HCC group, compared with CHB/LC and HD groups (all \( P<0.05 \), Figure S3). Using \( 2^{\Delta\Delta C_{q}} = 2.0 \) as a cutpoint,\(^{15,26} \) positivity rates in the HCC group for EpCAM (43.50%), CD133 (33.50%), CD90 (34.00%), and CK19 (29.00%) were significantly higher than those in CHB/LC and HD groups (all \( P<0.05 \), Figure S3). Diagnostic accuracies in using these four biomarkers were as follows: EpCAM, AUC=0.70 (95% CI 0.65-0.74); CD133, AUC=0.65 (95% CI 0.60-0.70); CD90, AUC=0.64 (95% CI 0.59-0.69); and CK19, AUC=0.64 (95% CI 0.59-0.69). Multivariate \( P \) values for all of four biomarkers were <0.05 by logistic regression (Table S2).

Consequently, a backward logistic regression model was applied to estimate
the risk of malignant (HCC) diagnosis for the training dataset, issuing the
following new variable of predicted probability (p) for HCC on the basis of an
equation derived from logistic regression (all HCC vs. control groups in training
set):

\[
\text{logit } (p=\text{HCC}) = -2.15 + 0.74 \times \text{EpCAM} + 0.65 \times \text{CD133} + 0.34 \times \text{CD90} + 0.99 \times \text{CK19}.
\]

Performance of the CTC detection panel in training set

In accordance with our logistic regression equation, a predicted probability was
registered for each member of the training set. These values were significantly
higher in patients with HCC (n=200) versus CHB/LC (n=101) or HD groups
(n=100) (both \(P<0.001\), Figure 2A). Further ROC analysis in patients with
HCC versus CHB/LC and HD showed optimal diagnostic cutoff value of 0.57
(criteria for optimal sensitivity plus specificity, optimal Youden index) for CTC
panel and 0.88 for AUC, with 72.5% sensitivity and 95.0% specificity,
compared with an AUC of 0.77 for AFP, with 57.0% sensitivity and 90.0%
specificity (Figure 3A, Table 2, cutoff value of AFP= 20 ng/ml). A greater
proportion of patients with HCC were positive on the basis of CTC panel, as
opposed to AFP (73.0% vs. 57.5%, cut off value for CTC load/5ml=0.57 and
AFP=20 ng/ml, Figure 2B), and similar CTC panel positivity rates were
observed in AFP-negative and AFP-positive patients with HCC (77.9% vs.
71.1%, Figure 2B). Furthermore, patients with early-stage HCC showed a
higher proportion of positive CTC panel results than for AFP (71.8% vs. 53.4%,
The CTC panel outperformed AFP as a biomarker in terms of differential diagnostic capability, yielding higher AUC, sensitivity, and specificity in HCC (n=200) and CHB/LC group (n=101) comparisons (Figure 3C, Table 2). In the CHB/LC group (n=101), 19.8% had above-threshold serum AFP levels, compared with only 9.9% on CTC panel screening, and only 3 of 20 AFP-positive (15.0%) CHB/LC subjects were positive by CTC panel screening (Figure 2D). Compared with AFP, the CTC panel also showed higher potential in diagnosing early-stage HCC (BCLC 0+A) (n=131) (Figure 3B, D). At differing BCLC stages, consistently accurate panel results were seen: stage 0, AUC=0.92 (95% CI 0.88-0.95); stage A, AUC=0.86 (95% CI 0.82-0.90); stage B, AUC=0.91 (95% CI 0.87-0.94); and stage C, AUC=0.86 (95% CI 0.82-0.91) (Table S3).

Additionally, we found that cancerous and non-cancerous states in AFP-negative patients were distinguished satisfactorily via the CTC panel (AUC=0.89, 95%CI 0.85-0.92; sensitivity, 77.7%; specificity, 95.0%; Table S4). More importantly, this level of performance was maintained in the early-stage HCC subgroup of AFP-negative patients (AUC=0.89, 95% CI 0.84-0.92; sensitivity, 75.4%; specificity, 95.0%; Table S4).

Validating the diagnostic potential of the CTC detection panel
We found that relative expression levels for all four markers were still significantly lower in HD (n=110), CHB/LC (n=100) and BHL groups (n=100) compared with HCC group (n=195) in validation set ($P<0.05$, Figure S4). When comparing HCC (including early-stage subset) with other three control groups (HD, CHB/LC and BHL), the similar differences in patterns of predicted probabilities were observed (Figure 2A). Relative to three control groups, AUC of the CTC panel was 0.93 (95% CI 0.90-0.95), with 82.1% sensitivity and 94.2% specificity for all HCC, and 0.93 (95% CI 0.90-0.96) with 85.1% sensitivity and 94.2% specificity for early-stage HCC (n=94), all surpassing the performance of serum AFP (Figure 3A, B, and Table 2). Using the 0.57 cut-off point for diagnosis, our CTC panel positivity rates remained high in the validation set (82.0%), including early-stage patients (85.1%), but were low in the CHB/LC group (8.0%) and BHL group (13.0%) regardless of AFP status (Figure 2 B, D and Figure S5). The panel diagnostic performance was outstanding in both negative and positive AFP subgroups (Table S4). The capacity of CTC panel to distinguish patients with HCC (even at early stage) from those with CHB/LC and BHL in validation set was also confirmed (Figure 3C, D). Meanwhile, the similar satisfactory diagnostic performances of CTC panel were observed in different BCLC stages (Table S3).

The clinical significance of our CTC panel in treatment response surveillance
The potential utility of the CTC panel in therapeutic response evaluation was further investigated in 60 resectable HCC patients. We found that the CTC load was decreased significantly after resection in 76.7% (46/60) of HCC patients with blood sample collection 1 month after surgery, and the positive rate decreased from 70.0% (42/60) to 31.7% (19/60) (Fig. 4A). With a median follow-up time of 25.8 months, 46.7% (28/60) of the patients suffered intrahepatic recurrence. On the basis of changes between preoperative and postoperative CTC level, we divided the 60 patients into three groups: I, persistent negative (n=18) at both points; II, preoperatively positive then postoperatively negative (n=23); and III, persistent positive (n=19). The recurrence rates for groups I-III were 11.1% (2/18), 47.8% (11/23), and 78.9% (15/19), respectively. Patients in group III showed a significantly higher recurrence rate than those in groups II and I (both \( P < 0.050 \), Fig. 4B).

The prognostic significance of the CTC panel in HCC patients undergoing resection

Based on X-Tile analysis, an optimal cutoff point of 0.80 (CTC load/5ml) for the CTC panel showed the most significant power to predict patients outcome in the training set (Fig. S6), and the HCC patients were stratified into CTC load/5ml ≤ 0.80 (CTC low) or CTC load/5ml > 0.80 (CTC high). In training set, with a median follow-up time of 25.2 months, 62.5% (75/195) of these patients suffered tumor recurrence. Patients with CTC load/5ml > 0.80 had significantly
shorter TTR (median, 27.7 months vs. not reached) and higher recurrence rates (49.1% vs. 24.7%) than those with CTC load $\leq 0.80$ ($P < 0.001$, Figure 4C). On multivariate analysis, the CTC panel was an independent prognostic factor for TTR (HR = 2.692, 95% CI, 1.617-4.483; $P < 0.001$, Table S5), and the similar results were also confirmed in the validation set (HR = 3.127, 95% CI, 1.360-7.190; $P = 0.007$, Figure 4C and Table S5). Furthermore, in AFP-negative and early-stage subgroups, patients with preoperative CTC load/5ml$>0.80$ also showed a relatively higher risk of developing postoperative tumor recurrence than those with CTC load/5ml$\leq 0.8$ (All $P < 0.05$, Figure 4D, E).
Discussion

Nucleic acid-based CTC detection methods have the advantages of high sensitivity and small sample volume required.\textsuperscript{28,29} Using an optimal multimarker qRT-PCR detection platform with negative enrichment strategy,\textsuperscript{15} the aim of this study was to evaluate expression patterns of putative CSC biomarkers in CTCs and investigate the diagnostic, prognostic values as well as treatment response evaluation of CTCs with stem-like features in HCC. Searching for appropriate mRNA marker expressed specifically by tumor cells is critical for the specificity and reliability of CTC detection. After screening the expression patterns of nine putative CSC biomarkers systematically, a CTC detection panel, including EpCAM, CD90, CD133, and CK19, was then constructed through a multivariate logistic regression model. We found the panel exhibited highly accurate in diagnosing HCC, especially in early-stage and AFP-negative diseases. Meanwhile, our CTC detection panel showed great significance in predicting early recurrence of HCC after resection as well as its potential in monitoring therapeutic response. We also found that the multi-marker CTC panel shows better AUC than single marker EpCAM in differentiate HCC and control groups (Figure S7 A), and the prognostic significance of CTC panel still retained in EpCAM\textsuperscript{-}subgroup (Figure S7 B). These results indicating that our multi-marker CTC outperforms EpCAM alone in HCC diagnostic and prognostic evaluation. Thus, our study implied that this panel could serve as a useful complement tool for early diagnosis, outcome
prediction and treatment evaluation, which is urgently need in HCC management.

CSCs are thought to be responsible for tumor recurrence and metastasis,\(^3^0\) and these cells seem more capable of vascular invasion, tending to circulate in even early-stage tumors.\(^3^1\) Although both CSCs and mature cancer cells can migrate into the blood stream, CSCs are more prone to survive in the circulation and deposit in distant organs or re-circulate back to the liver remnant.\(^1^2\) For these reasons, we focused the CTC subpopulations with stem-like phenotypes instead of whole CTC population detection for HCC diagnosis, prognosis and therapeutic evaluation. Given the documented heterogeneity of CTCs in HCC and the results of our previous study using a single marker,\(^1^5\) a multimarker qRT-PCR assay for CTC detection was conducted, and nine putative CSC markers were chosen for the biomarkers screening of CTCs with stem-like cell features (see references of ours and others)\(^1^1-1^9\) and only four were finally included for constructing our CTC detection panel.

Serum AFP has been widely used as a diagnostic and screening marker of HCC. However, approximately 30-40% of patients with HCC are AFP negative,\(^3^2,3^3\) and elevated AFP concentrations have been reported in 11-58% of patients with chronic hepatitis or cirrhosis, without HCC.\(^3^4,3^5\) Our data indicated that the differential diagnostic capability of our CTC panel performed much better than serum AFP in both training and validation sets (Figure 2, 3
and Table 2). Furthermore, the excellent diagnostic performances of our panel were sustained in AFP-negative subsets of HCC (Table S4). Currently, imaging remains an important tool for diagnosis and staging of HCC. Nevertheless, approximately 30% of patients with HCC lack typical imaging manifestations, possibly because the diagnostic accuracy of imaging is largely affected by tumor size. Our CTC panel had significant value in diagnosing patients with early-stage HCC, including those with single tumor of ≤ 2 cm. Indeed, small-sized lesions of HCC could be detected in subjects at risk, supporting use of our CTC screening panel in this setting. More importantly, when patients at risk without visible hepatic lesion are positive for CTC panel detection, we should make a close follow-up to check tumor existence for timely intervention. Thus, our findings underscore the promise of our CTC panel as a novel biomarker for early diagnosing HCC and complementing current diagnostic protocols.

According to the hypothesis that CTCs might be the “seeds” of tumor metastasis and recurrence, the prognostic significance of the CTC panel was further confirmed in HCC patients undergoing surgery and high recurrence rate in patients with high CTC load was observed. Our CTC detection panel might serve as a novel indicator reflecting the micrometastatic status which could not be detected by routine image tools, and estimating the recurrence risk of HCC patients in a real-time manner. Meanwhile, the decrease of CTC load was observed soon after resection, and patients with
persistently high CTC load postoperatively showed a propensity of increased
tumor recurrence, and this suggested that our CTC panel might be a surrogate
indicator for surveillance of the treatment response during HCC management.
In addition, further study focus on these CTC subpopulations (such as
EpCAM+, CD90+, CD133+, and CK19+) will be present more information for
metastatic mechanism as well as related therapeutic targets.
Epithelial-mesenchymal transition (EMT) is essential for circulatory invasion by
tumor cells, which become CTCs, and EMT markers are also candidate
indices of CTCs. We evaluated expression patterns of two major EMT
markers (E-cadherin and Vimentin) in 89 patients with HCC patients and in 70
HD subjects, and there had no significant difference in two groups (Figure S8).
To our knowledge, this is the first large-scale, multicenter study to report the
utility of CTCs detection in HCC diagnosis, prognosis as well as therapeutic
evaluation, using both training and validation sets. However, HCC stemmed
from cirrhosis or HBV infection in all patients we recruited, unlike conditions in
the USA, Europe, and Japan. Thus, the clinical relevance of our CTC panel
should be validated in patients from other geographic areas. In addition, the
clinical significances of this CTC panel were only evaluated in HCC patients,
the application in other solid tumors needed to be further confirmed.
In conclusion, we have generated a multimarker CTC detection panel with high
sensitivity and specificity, capable of differentiating patients with HCC from
healthy control, patients with CHB, LC, and BHL. More importantly, this CTC
panel may have merit as a tool for providing information about the patient’s current disease state and assessing therapeutic response in a real-time manner, which might play an important role in personalized therapy for patients with HCC in the future.
Acknowledgments

The authors thank the participating patients for the source of clinical blood samples.
References


23. Forner A, Gilabert M, Bruix J, Raoul JL. Treatment of intermediate-stage


## Tables

### Table 1. Clinicopathologic characteristics of patients with HCC and CHB/LC groups in training and validation sets

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<th>Training Set</th>
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<td><strong>AFP (ng/ml)</strong></td>
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<td></td>
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<tr>
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<td>( &gt; 20 )</td>
<td>115 (57.50)</td>
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<td><strong>Age (y)</strong></td>
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<td>( \leq 50y )</td>
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<td>117 (58.50)</td>
<td>121 (62.05)</td>
<td></td>
</tr>
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<tr>
<td>Female</td>
<td>47 (23.50)</td>
<td>34 (17.44)</td>
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<tr>
<td>Male</td>
<td>153 (76.50)</td>
<td>121 (62.05)</td>
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<tr>
<td><strong>HBsAg</strong></td>
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<td>47 (23.50)</td>
<td>34 (17.44)</td>
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<td>121 (62.05)</td>
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<td>Multiple</td>
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<td>131 (65.50)</td>
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<td><strong>CHB/ LC group</strong></td>
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<td><strong>AFP (ng/ml)</strong></td>
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**Abbreviations:** HCC, hepatocellular carcinoma; CHB, chronic hepatitis B infection; LC, liver cirrhosis; AFP, alpha-fetoprotein; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; N.A., not applicable.
Table 2. Performance of the CTC panel, serum AFP, or both in diagnosing HCC*  

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<th>Validation (n=505)</th>
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<tr>
<td></td>
<td>AUC (95%CI)</td>
<td>Sen (%)</td>
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<td>Sp (%)</td>
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<td>PLR</td>
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<td>HCC vs. CHB, LC, and HD</td>
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<tr>
<td>CTC</td>
<td>0.88 (0.84-0.91)</td>
<td>72.5</td>
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<tr>
<td>AFP</td>
<td>0.77 (0.73-0.81)</td>
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<td></td>
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<tr>
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<td>95.0</td>
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<tr>
<td>HCC vs. CHB and LC</td>
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<tr>
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<td>92.0</td>
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<td>Early-stage HCC vs. CHB, LC, and HD</td>
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<tr>
<td>CTC</td>
<td>0.87 (0.83-0.90)</td>
<td>71.8</td>
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<td>AFP</td>
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<td>Early-stage HCC vs. CHB and LC</td>
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<td>0.3</td>
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**Abbreviations:** HCC, hepatocellular carcinoma; CTC, circulating tumor cell; AFP, α-fetoprotein; AUC, area under the ROC curve; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; LC, liver cirrhosis; CHB, chronic hepatitis B infection; BHL, benign hepatic lesion; HD, healthy donor. *Diagnostic cut-points of CTC panel and serum AFP were 0.57 and 20 ng/ml, respectively.
Figure Legends

Figure 1. Flowchart of study design

Figure 2. Distributions of predicted probabilities and positivity rates for the CTC diagnostic panel in training and validation sets

(A) Distributions for predicted probabilities of the CTC panel in training (left) and validation (right) groups; (B) Positivity rates for the CTC panel, serum AFP, or both and for the CTC panel stratified by AFP status in all HCC patients of training (left) and validation (right) groups; (C) Positivity rates for the CTC panel, serum AFP, or both and for the CTC panel stratified by AFP status in patients with early-stage HCC of training (left) and validation (right) groups; and (D) positivity rates for the CTC panel and serum AFP and for the CTC panel with AFP-positivity in patients with chronic HBV infection and/or cirrhosis of training (left) and validation (right) groups.

Figure 3. ROC analysis of the CTC panel performance in diagnosing HCC for training and validation sets

ROC curves for: (A) the CTC diagnostic panel and serum AFP in all patients with HCC versus all controls of training (left) and validation (right) groups; (B) the CTC panel and serum AFP in patients with early-stage HCC versus all controls of training (left) and validation (right) groups; (C) the CTC panel and serum AFP in all patients with HCC versus patients at risk of HCC of training...
(left) and validation (right) groups; and (D) the CTC panel and serum AFP in patients with early-stage HCC versus patients at risk of HCC of training (left) and validation (right) groups.

Figure 4. The prognostic significance of the CTC panel in HCC patients after operation. (A) The CTC positivity rates before and after curative resection; (B) The prognostic significance of CTC loads with respect to time to recurrence in patients with persistent positive CTC, conversion of CTC from positive to negative, and persistent negative CTC; (C) The Kaplan-Meier analysis of TTR for the CTC panel in training (left) and validation (right) groups; (D) The Kaplan–Meier analysis of TTR for the CTC panel in BCLC0+A subgroups in training (left) and validation (right) groups; (E) The Kaplan–Meier analysis of TTR for the CTC panel in AFP ≤ 20 subgroups in training (left) and validation (right) groups.

(CTC high represents CTC load/5ml > 0.80, CTC low represents CTC load/5ml ≤ 0.80)
Figure 1

Number of individuals informed consents (n=1006)

Screening set (n=100)
Zhongshan Hospital
(HCC=50, Healthy=50)

Training set (n=401)
Zhongshan Hospital
Shanghai Public Health Clinical center
Longhua Hospital

Validation set (n=505)
Zhongshan Hospital
Shanghai Public Health Clinical center
Shanghai Tumor center

100 tests by optimized platform and markers screened out
(EpCAM, CD133, CD90, CK19)

qRT-PCR measurement and cutoff value assessed

Diagnostic exploration
HCC (n=200)

Prognostic exploration
Resectable HCC (n=195)

CHB/LC (n=101) Healthy (n=100)

TACE (n=5)

Diagnostic validation
HCC (n=195)

Prognostic validation
Resectable HCC (n=130)

CHB/LC (n=100) BHL (n=100) Healthy (n=110)

TACE (n=65)
Figure 3

A

Training

Sensitivity vs. 1 - Specificity

HCC vs. CHB/LC+HD

- CTC AUC=0.88
- AFP AUC=0.77
- Reference Line

Validation

Sensitivity vs. 1 - Specificity

HCC vs. CHB/LC+BHL+HD

- CTC AUC=0.93
- AFP AUC=0.80
- Reference Line

B

Sensitivity vs. 1 - Specificity

Early-HCC vs. CHB/LC+HD

- CTC AUC=0.87
- AFP AUC=0.74
- Reference Line

Early-HCC vs. CHB/LC+BHL+HD

- CTC AUC=0.93
- AFP AUC=0.75
- Reference Line

C

Sensitivity vs. 1 - Specificity

HCC vs. CHB/LC

- CTC AUC=0.87
- AFP AUC=0.72
- Reference Line

HCC vs. CHB/LC+BHL

- CTC AUC=0.93
- AFP AUC=0.79
- Reference Line

D

Sensitivity vs. 1 - Specificity

Early-HCC vs. CHB/LC

- CTC AUC=0.85
- AFP AUC=0.68
- Reference Line

Early-HCC vs. CHB/LC+BHL

- CTC AUC=0.93
- AFP AUC=0.74
- Reference Line
Figure 4

A

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<th>Post</th>
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<td>Negative (CTC load/5ml ≤ 0.57)</td>
<td>30.00%</td>
<td>70.00%</td>
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<tr>
<td>Positive (CTC load/5ml &gt; 0.57)</td>
<td>68.33%</td>
<td>31.67%</td>
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<td>(n=60)</td>
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B

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<td>Persistent negative (n=18)</td>
<td>Persistent positive (n=19)</td>
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<td>II: Positive to negative (n=23)</td>
<td>Persistent positive (n=19)</td>
</tr>
<tr>
<td>I vs. II P=0.0167</td>
<td>I vs. III P=0.0001</td>
</tr>
<tr>
<td>II vs. III P=0.0059</td>
<td></td>
</tr>
</tbody>
</table>

C

CTC low (CTC load/5ml ≤ 0.80, n=85) vs. CTC high (CTC load/5ml > 0.80, n=110)

P < 0.001

D

CTC low (CTC load/5ml ≤ 0.80, n=63) vs. CTC high (CTC load/5ml > 0.80, n=68)

P = 0.013

BCLC 0+A

E

CTC low (CTC load/5ml ≤ 0.80, n=33) vs. CTC high (CTC load/5ml > 0.80, n=50)

P = 0.025

AFP ≤ 20ng/ml

BCLC 0+A

AFP ≤ 20ng/ml
Circulating tumor cells with stem-like phenotypes for diagnosis, prognosis and therapeutic response evaluation in hepatocellular carcinoma

Jia Fan, Wei Guo, Yun-Fan Sun, et al.

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