Personalized Medicine and Imaging

Clinical Significance of EpCAM mRNA-Positive Circulating Tumor Cells in Hepatocellular Carcinoma by an Optimized Negative Enrichment and qRT-PCR-Based Platform

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Abstract

Purpose: This study aimed to construct a novel platform for the detection of circulating tumor cells (CTC) in patients with hepatocellular carcinoma (HCC) and to investigate the clinical significance of epithelial cell adhesion molecule mRNA-positive (EpCAM mRNA+) CTCs using this platform.

Experimental Design: An optimized platform for CTC detection was constructed by evaluating different negative enrichment, mRNA isolation, and cDNA synthesis procedures and compared with the CellSearch system. A total of 299 patients with HCC were recruited into this prospective study; of these, 157 who received curative resection, 76 who received transcatheter arterial chemoembolization (TACE), and 66 who received radiotherapy were tested using our platform. The diagnostic value of EpCAM mRNA+ CTCs was investigated in 122 patients with HCC who underwent resection and 120 control subjects.

Results: The optimized negative enrichment and quantitative real-time PCR (qRT-PCR)-based CTC detection platform had high sensitivity, specificity, and reproducibility and a low sample volume requirement. This platform showed a potential diagnostic value in patients with HCC and exhibited 76.7% consistency with the CellSearch system (r = 0.54, P < 0.050). Pretreatment CTC level showed prognostic significance in patients with HCC treated with resection, TACE, and radiotherapy (all P < 0.050). Most of the patients showed a decrease in CTC levels after treatment that reflected tumor response. In contrast, patients with an increased CTC level showed disease progression after treatment.

Conclusions: We established an optimized platform based on negative enrichment and qRT-PCR for highly sensitive, specific, and reproducible CTC detection. This platform might be clinically useful in auxiliary diagnosis, treatment response assessment, and early decision-making to tailor the most effective antitumor strategies.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with an increasing incidence in recent years and dismal outcomes (1). Surgery remains the most effective treatment with curative potential, but only approximately 10% to 20% of patients with HCC are eligible for surgical intervention due to the lack of an effective means for early diagnosis (2). For the remaining patients, transcatheter arterial chemoembolization (TACE), radiotherapy, or sorafenib are common treatment choices (3). The lack of effective methods for timely diagnosis and monitoring anticancer treatment response is the main obstacle preventing improvement of overall survival (OS) of patients with HCC (4). At present, serologic tumor markers, clinicopathologic parameters, and radiologic modalities are used in routine clinical practice for management of patients with HCC; however, none of these approaches can provide comprehensive information covering HCC diagnosis, prognosis prediction, and effective surveillance of therapeutic response. Therefore, there is an urgent need for a reliable and versatile method that integrates early detection of HCC, predicting risk of recurrence or metastasis, and monitoring antitumor treatment response in a real-time manner (5).

Recent data have provided evidence that the generation of circulating tumor cells (CTCs) is an early event in tumor progression and that CTCs are directly involved in the
metastatic cascade (6–13). CTCs expressing epithelial cell adhesion molecule (EpCAM) can be detected in patients with HCC and is an independent prognostic factor of time to recurrence (TTR) in these patients. However, detection of CTCs remained costly via CellSearch system, and clinical significance of these CTCs remained unclear in patients with unresectable HCC. Therefore, we established a novel optimized negative enrichment and quantitative real-time PCR (qRT-PCR)–based CTC detection platform that exhibits high sensitivity, specificity, and reproducibility with a small sample volume requirement for patients with HCC. On the basis of this platform, we comprehensively evaluate the clinical significance of EpCAM mRNA-positive (EpCAMmRNA+) CTCs in patients with HCC, and showed that detection of EpCAMmRNA+ CTCs by our qRT-PCR platform might serve as a novel indicator for the risk of recurrence or progression in patients with HCC and a promising surrogate tool for surveillance of the therapeutic response instead of radiologic scans with the benefits of increased convenience and safety.

Translational Relevance
Circulating tumor cells (CTC) expressing epithelial cell adhesion molecule (EpCAM) can be detected in patients with HCC using the CellSearch system and are an independent prognostic factor of time to recurrence (TTR) and OS in these patients (14, 15). However, the clinical significance of CTC detection as an accurate prognostic tool and/or a real-time indicator for treatment response in patients with HCC has not been comprehensively evaluated.

The two major assays that are widely used for CTC detection are immunomagnetic assays represented by the CellSearch system and quantitative real-time PCR (qRT-PCR). Compared with the CellSearch system, CTC detection by qRT-PCR provides accurate and quantitative information on gene expression and has the advantage of improved sensitivity, versatility, and cost-effectiveness (16–18). More importantly, molecular characterization of CTC by qRT-PCR analysis may offer new perspectives regarding the clarification of molecular mechanism of metastasis, analysis of the association of CTC molecular profiles with treatment outcomes, and identification of potential therapeutic targets (19–23). Moreover, when combined with negative enrichment to reduce background signals by depleting leukocytes from blood, the qRT-PCR assay shows impressive sensitivity and specificity for CTC detection (24–26).

In light of these considerations, we constructed a novel optimal qRT-PCR–based CTC detection platform based on currently available commercial kits, and then conducted a prospective single-center study to explore the prognostic significance and dynamic changes of EpCAM+ CTCs in patients with HCC undergoing surgery, TACE, or radiotherapy.

Patients and Methods
Study design
From March 2012 to October 2013, 299 patients with HCC were recruited into this prospective study that aimed to construct a negative enrichment and qRT-PCR–based CTC detection platform and investigate the clinical significance of EpCAMmRNA+ CTCs. HCC was defined on the basis of pathologic diagnosis, biopsy, ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) characteristics, and α-fetoprotein (AFP) serology according to the American Association for Study of Liver Disease guidelines (27). Among these patients, 157 received curative resection (defined as complete macroscopic removal of tumor; ref. 28), 76 received TACE, and 66 received radiotherapy treatment. The patients that received TACE or radiotherapy had Barcelona Clinical Liver Cancer (BCLC) B or C stage disease and both treatments targeted intrahepatic lesions. The enrolled individuals were separated into three independent cohorts for clinical evaluation (Fig. 1). In addition, 71 healthy donors, 24 patients with benign tumor, and 25 patients with chronic Hepatitis B and/or liver cirrhosis but without a history of malignancy were enrolled as negative controls.

Preoperative CTC detection was performed in 222 patients with HCC undergoing resection (n = 122), TACE (n = 56), or radiotherapy (n = 44). A second group of 77 patients (35 treated with resection, 20 with TACE, and 22 with radiotherapy) underwent CTC detection at pretreatment and 1 month after treatment.

Posttreatment surveillance was performed as described previously (29). Radiologic interpretation was performed by two certified radiologists using modified Response Evaluation Criteria in Solid Tumors (mRECIST; ref. 30). For evaluation of prognostic value, follow-up was completed in March 2014. TTR was defined as the interval between surgery and the diagnosis of any type of recurrence, with intrahepatic recurrence and extrahepatic metastasis defined as the end points for the TTR (31). Progression-free survival (PFS) was defined as the time between the date of receiving treatment and the date of clinical disease progression or the date of the last follow-up visit if progression did not occur during the follow-up (32). Approval for the use of human subjects was obtained from the research ethics committee of Zhongshan Hospital (Shanghai, PR China), and informed consent was obtained from each individual enrolled in this study.

Cell line
The human HCC cell line Hep3B was purchased from and authenticated by the ATCC (ATCC HB-8064; ref. 14). It was passaged for less than 6 months after receipt and used in spiking experiments of the current study. The cell line was characterized by the cell bank based on cell morphology, post-freeze viability, isoenzyme analysis, DNA fingerprinting analysis, mycoplasma contamination test, and bacterial and fungal contamination (33). The cell line was maintained in DMEM (Gibco) supplemented with 10% FBS.
(Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin, at 37 °C under 5% CO2 in incubator. It was routinely screened for presence of mycoplasma (Mycoplasma Detection Kit, Roche Diagnostics) during the study period.

**Optimized CTC enrichment method**

Hep3B cells were stained with Mito Tracker Orange (Invitrogen) and different numbers of stained cells (10, 10^2, 10^3, and 10^4) were spiked into 5-mL samples of blood from a normal donor. These samples were processed by two negative enrichment methods: RosetteSep Human CD45 Depletion Cocktail (StemCell) and magnetic activating cell separation (MACS) HEA CD45 microbeads (Miltenyi Biotech). After enrichment, the cell suspension was observed under a LX-71 fluorescence microscope (Olympus) and the recovery rate was calculated.

**Optimal RNA isolation method**

To determine the optimal method for RNA isolation, we compared the RNA yield from two commonly used commercial RNA isolation kits: TRIzol (Invitrogen) and RNeasy Mini Kit (Qiagen). We spiked 1, 5, 10, 50, and 100 Hep3B cells into 5 mL PBS and extracted total RNA using the two kits according to the manufacturers’ instructions. Reverse transcription and qRT-PCR was performed using the same protocol for all samples. The method with the highest sensitivity was selected as the optimal RNA isolation method.

**Optimal cDNA synthesis method**

To determine the optimal method for cDNA synthesis, we resuspended 1, 5, 10, 50, and 100 Hep3B cells in 5 mL PBS. After extraction of total RNA with the same method, equal quantities of RNA were reverse transcribed using SuperScript III First-Strand Kit (Invitrogen) or Quant iTect Reverse Transcript Kit (Qiagen) according to the manufacturers’ instructions and qRT-PCR was performed. The method with the highest sensitivity was selected as the optimal cDNA synthesis method.

**qRT-PCR**

Total RNA was extracted and reverse transcribed into cDNA. qRT-PCR analysis of EpCAM and \( \beta \)-actin transcripts was performed using the Light Cycler 480 platform (Roche Diagnostics) with fluorescent TaqMan methodology. The forward primer 5’-TCGCGTGCCGGGCCTGC-3’, the reverse primer 5’-TGTAGTITTCACAGACATTCCTCCT-3’, and the probe [6FAM] ACGGCACCTTTGCACGGCA-MRA were used for analysis of EpCAM expression. The forward primer 5’-GCGATGGGGCCACAGCT-3’, the reverse primer 5’-TCGCGGACAGCAATTTGTTG-3’, and the probe [6FAM] -ATCACTGCCCTGCGACCAGCATA-MRA were used for analysis of \( \beta \)-actin expression. All primers and probes were designed and synthesized by the Life Technologies Corporation (Invitrogen). PCR reactions were performed with Platinum qPCR SuperMix-UDG (Invitrogen) using the following conditions: 2 minutes at 50 °C and 2 minutes at 95 °C, followed by 45 cycles of 95 °C for 3 seconds and 60 °C for 30 seconds. Each sample was analyzed in triplicate. Gene expression levels were calculated according to the following equation: 2\(^{\Delta C_q}\) \( \Delta C_q = C_q (\text{target}) - C_q (\beta\text{-actin}) \).

**Overall performance evaluation of the optimized protocol**

After construction of the optimal platform, intrarun and interrun precision was evaluated by repeated experiments, and sensitivity and linearity studies were conducted using a continuous dilution test. Details are given in Supplementary Materials and Methods.

**qRT-PCR analysis of clinical samples**

Gene expression levels were calculated according to the following equation: 2\(^{\Delta \Delta C_q}\) \( \Delta \Delta C_q = C_q (\text{target}) - C_q (\beta\text{-actin}) \). The qRT-PCR results of clinical samples were analyzed by relative quantification using the 2\(^{\Delta \Delta C_q}\) algorithm, which gives the fold expression relative to a calibrator (average \( \Delta C_q \) of healthy volunteers). The cut-off value was set at 2.0 based on a previous study (34). Samples were classified as positive for EpCAM gene expression if the value
of $2^{-\Delta C_{q}}$ was higher than the cut-off value. Samples with undetectable EpCAM ($C_{q}=45$) were assigned a $2^{-\Delta C_{q}}$ value of 0.

Enumeration and detection of CTCs using the CellSearch system

EpCAM$^+$ CTC analysis was performed using CellSearch (Veridex) as previously described (25). Results of CTC enumeration were expressed as the number of cells per 7.5 mL of blood (CTC$^{7.5}$).

Statistical analysis

Statistical analyses were performed using SPSS 19.0 for Windows (IBM). Experimental values are presented as mean ± SEM. χ$^2$ tests, Fisher exact probability test, and Student’s $t$ test were used for comparison between groups, as appropriate. If variances within groups were not homogeneous, the nonparametric Mann–Whitney $U$ test or Wilcoxon signed-rank test was used. A receiver operating characteristics (ROC) curve was used to evaluate the diagnostic value of CTCs. The relationship between TTR or PFS and CTC was analyzed by Kaplan–Meier survival curves and the log-rank test. A P value <0.050 was considered statistically significant.

Results

Patient characteristics

A total of 299 patients who had been pathologically or clinically diagnosed with HCC were enrolled in this study from March 2012 to October 2013. The clinical characteristics of the 299 patients are summarized in Table 1.

<table>
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<th>Table 1. Clinical characteristics of HCC patients and correlation with EpCAM$^{mRNA+}$ CTCs</th>
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Abbreviations: ALT, alanine transaminase; HBsAg, Hepatitis B surface antigen; N.A., not applicable.

* Fisher exact test.

* -” represents negative for EpCAM$^{mRNA+}$ CTCs.

* “+” represents positive for EpCAM$^{mRNA+}$ CTCs.
Comparison of CTC enrichment protocols

First, we compared EpCAM mRNA expression of spiked Hep3B cells using Ficoll, MACS, and RosetteSep enrichment methods. EpCAM mRNA expression was detected in cells processed by the Ficoll procedure when no tumor cells were added, whereas no signal was detected by the two negative selection methods. Among the three methods, the relative EpCAM mRNA expression for the same number of spiked tumor cells was highest using RosetteSep (Fig. 2A).

Next, we spiked 5 mL of blood from a healthy volunteer with $10^0$, $10^1$, and $10^2$ stained Hep3B cells to test the recovery rate of the two negative enrichment methods. The recovery rate was calculated by the formula: recovery rate (%) = [number of cells recovered/number of cells spiked] × 100%. Typical images of negative selection are shown in Supplementary Fig. S1. For $10^0$, $10^1$, and $10^2$ cells, the recovery rate of MACS microbeads was significantly lower than that of RosetteSep (mean recovery rate, 3.1 vs. 58.3, 24.8 vs. 68.3, 51.7 vs. 71.9, and 66.9 vs. 81.9, respectively, all $P<0.050$; Fig. 2A). The RosetteSep method was therefore selected as the optimal enrichment method.

Comparison of RNA isolation and cDNA synthesis methods

RNA was isolated from a series dilution of Hep3B cells (1, 5, 10, 50, 100 cells) using TRIZol (Invitrogen) and the RNeasy Mini Kit (Qiagen). After reverse transcription, the sensitivity of each method was determined by qRT-PCR. EpCAM mRNA of a single Hep3B cell could be detected using the RNeasy Mini Kit, whereas no EpCAM mRNA signal was detected for 100 Hep3B cells using TRIZol reagent. Similarly, the $\beta$-actin mRNA of a single Hep3B cell could be detected using RNeasy, whereas with TRIZol reagent the minimum number of Hep3B cells for detection was 50 (Supplementary Table S1). Next, RNA from 1, 5, 10, 50, and 100 Hep3B cells was isolated using an RNeasy Mini

Figure 2. Construction of optimized CTC detection platform. A, relative EpCAM mRNA expression of spiked Hep3B cells measured by three methods (up), and recovery rate of two negative enrichment methods for CTCs (down). B, flowchart of the optimized platform. C, typical amplification curves and regression curve of the linearity experiment. Hep3B cells (1, 5, 10, 50, and 100 cells) were serially diluted in PBS and detected using the optimized protocol. The relative EpCAM expression was calculated using the $2^{-\Delta\Delta C_T}$ method and the curve was generated by the least square method. D, comparison of our optimized platform with the CellSearch system. (** represents significant difference between groups).
Kit and reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) or SuperScript III First-Strand (Invitrogen). qRT-PCR was performed to determine the sensitivity of each method. The β-actin gene could be detected using both cDNA synthesis methods for all cell dilutions tested. EpCAM gene expression could be detected in all dilutions using the Qiagen method, whereas for the Invitrogen cDNA synthesis kit the lowest cell number that allowed EpCAM detection was 100 (Supplementary Table S1).

**Construction of the optimized procedure for CTC detection**

On the basis of the above data, we established an optimized procedure for CTC detection in patients with HCC as follows: enrichment of CTCs in peripheral blood of patients with HCC using RosetteSep, isolation of mRNA using RNeasy Mini Kit, synthesis of cDNA using the Quantitect Reverse Transcription Kit, and qRT-PCR with the TaqMan Probe approach (Fig. 2B).

We performed repeat experiments to evaluate the precision of the protocol. Evaluation of intrarun and interday precision is illustrated in Supplementary Table S2 and the amplification curve is shown in Supplementary Fig. S2A and S2B. The results showed high precision of the protocol and excellent repeatability of the platform. We next spiked 10 mL of peripheral blood from a healthy volunteer with a dilution series of Hep3B cells (0, 1, 5, 10, 50, 100 cells) to evaluate the sensitivity and specificity of the established protocol. A single tumor cell in 10 mL peripheral blood could be detected by the optimized procedure with high sensitivity and specificity. Moreover, relative EpCAM mRNA expression was significantly correlated with the number of spiked tumor cells ($R^2 = 0.99$, Fig. 2C).

**Comparison between our optimized CTC detection platform and the CellSearch system**

As CellSearch is the only method currently approved by the U.S. Food and Drug Administration for CTC detection, we compared our optimized CTC detection platform with the CellSearch platform. Seventeen patients were enrolled in this experiment and paired peripheral blood samples (7.5 mL for CellSearch, 5 mL for our platform) were collected from each patient before resection. The comparison was performed as a double blind trial in two different clinical laboratories. Using the CellSearch system, the number of CTCs in samples from patients with HCC ranged from 1 to 5 and 52.9% (9/17) of patients were CTC-positive. Using our qRT-PCR-based detection platform, 41.2% (7/17) of patients had detectable EpCAM mRNA+ CTCs. The results of our optimized platform and the CellSearch system were consistent in 76.7% (13/17) of the paired samples ($r = 0.55$, $P < 0.050$; Fig. 2D).

**Diagnostic value of EpCAM mRNA+ CTCs detected by the optimized platform in patients with HCC**

Using our optimized qRT-PCR–based platform, the diagnostic value of EpCAM mRNA+ CTC was further investigated in 122 patients with HCC receiving resection, 71 healthy volunteers, 25 patients with hepatitis B, and/or cirrhosis, and 24 patients with benign tumor (relative EpCAM expression: 0–39.4, 0–1.9, 0–2.3, 0–3.8, respectively). The positive rates of EpCAM mRNA+ CTCs in the healthy control, hepatitis B and/or cirrhosis, benign tumor, and HCC groups were 0.0%, 8.0%, 8.3%, and 42.6%, respectively. Compared with the other three groups, the rate of detectable EpCAM mRNA+ CTCs was significantly higher in the HCC group ($P < 0.050$, Fig. 3A). The diagnostic value of EpCAM mRNA+ CTCs in HCC was evaluated, and the area under curve (AUC) for EpCAM mRNA+ CTCs in discrimination between patients with HCC and the other three groups was 0.697, with sensitivity of 42.6% and specificity of 96.7%. When combined with AFP level, the diagnostic value of CTCs was significantly improved and the AUC was 0.857 with a sensitivity of 73.0% and specificity of 93.4% (Fig. 3B).

**Dynamic changes and prognostic potential of EpCAM mRNA+ CTCs detected by the optimized platform in patients with resectable HCC**

The prognostic significance of EpCAM mRNA+ CTC in patients with resectable HCC was further investigated. We followed these 122 resectable patients over a median follow-up time of 15.3 months. During this time, 40.2% (49/122) of these patients suffered recurrence. Patients with preoperative detectable EpCAM mRNA+ CTCs had significantly shorter TTR (median, 10.9 months vs. not reached) and higher recurrence rates (59.6% vs. 25.7%) than those without detectable EpCAM mRNA+ CTCs ($P < 0.001$, Fig. 3C). In multivariate analysis, EpCAM mRNA+ CTC was the only independent prognostic factor for TTR [HR = 2.9; 95% confidence interval or CI, 1.6–5.3; $P < 0.001$, Supplementary Table S5].

Another 35 patients were recruited to investigate the dynamic changes in EpCAM mRNA+ CTCs during the perioperative period. Blood samples were collected before and 1 month after surgery (relative EpCAM expression: 0–22.6 for pretreatment; 0–8.5 for posttreatment). Compared with pretreatment, the loads of EpCAM mRNA+ CTCs decreased significantly after resection (48.6% vs. 17.1%, $P = 0.005$, Fig. 3D). Among these patients, 13 patients with CTC positivity before resection changed to a negative status 1 month after operation, 4 patients remained CTC-positive, and 2 CTC-negative patients tested positive after the operation. We followed these patients over a median time of 11 months. During this time, 7 of 35 patients suffered intrahepatic recurrence. On the basis of changes between preoperative and postoperative CTCs, the 35 patients were divided into four groups: I, persistent positive ($n = 4$) at both points; II, preoperatively positive then postoperatively negative ($n = 13$); III, negative then positive ($n = 2$); and IV, persistent negative ($n = 16$). The recurrence rates for groups I–IV were 75.0%, 7.7%, 50.0%, and 12.5%, respectively. Patients in group I showed significantly higher recurrence rates than those in groups II and IV (both $P < 0.050$; Fig. 3D).
Figure 3. Clinical evaluation of optimized CTC detection platform. A, distribution of EpCAM expression in healthy volunteers (○) and patients with Hepatitis B (●), benign tumor (△), and HCC (□) (up), and CTC-positive rates of healthy volunteers and patients with hepatitis B, benign tumor, or HCC (down) after 2^ΔΔCt algorithm transformation. The cutoff to discriminate between CTC-positivity and -negativity was set at 2.0. B, ROC plots for EpCAM mRNA detection and combined EpCAM mRNA and serum AFP detection in discriminating HCC from non-HCC groups. C, Kaplan-Meier analysis of patients with HCC receiving curative resection treatment according to relative pretreatment EpCAM expression <2.0 or ≥2.0 (2^ΔΔCt algorithm transformation). D, dynamic changes in relative EpCAM mRNA expression in patients receiving curative resection (left), CTC-positive rates of patients before and after curative resection (middle), and prognostic significance of CTC load with respect to time to recurrence in patients with persistent positive CTC, conversion of CTC from positive to negative, conversion of CTC from negative to positive, and persistent negative CTC (right). (*** represents significant difference between groups).
The prognostic potential of EpCAM mRNA+ CTCs in patients with unresectable intermediate-advanced HCC

To further explore the prognostic significance of EpCAM mRNA+ CTCs in patients with unresectable intermediate-advanced HCC, we recruited 100 patients with HCC undergoing TACE (n = 56) or radiotherapy (n = 44; relative EpCAM expression: 0–14.3 for TACE; 0–15.2 for radiotherapy). We found that patients in the radiotherapy group with disease progression showed higher relative EpCAM expression (2^ΔΔCq) than patients without disease progression (mean 5.7 vs. 2.4, P = 0.004; Fig. 4A). Similar to the result in radiotherapy group, a higher rate of EpCAM expression was observed in patients with tumor progression in TACE group (mean 4.0 vs. 2.5, P = 0.022; Fig. 4A).

The median follow-up time was 9.8 months for patients undergoing TACE and 10.5 months for patients undergoing radiotherapy. In the TACE group, progression was observed in 22 of 34 patients with pretreatment EpCAM mRNA+ CTCs, whereas only 5 of 22 patients without pretreatment
EpCAM mRNA+ CTCs developed tumor progression (64.7% vs. 22.7%; \( P = 0.004 \); Fig. 4A and B). In the radiotherapy group, 10 of 20 patients with pretreatment EpCAM mRNA+ CTCs developed tumor progression, and only 3 of 24 patients without CTCs showed tumor progression (50.0% vs. 12.5%; \( P = 0.006 \); Fig. 4A and B). In Cox regression analysis, pretreatment EpCAM mRNA+ CTC was proven to be the independent prognostic factor for PFS in both TACE and radiotherapy groups (TACE: HR = 3.8; 95% CI, 1.4–10.0; \( P = 0.008 \); radiotherapy: 5.1; 1.4–18.5; \( P = 0.014 \); Supplementary Table S5).

The prognostic significance of pretreatment CTC level within BCLC subgroups was further investigated. Patients who were positive for CTCs before treatment had a higher risk of developing tumor progression than those without CTCs in both the BCLC B group (55.2% vs. 5.3%, \( P < 0.001 \)) and BCLC C group (60.0% vs. 25.9%, \( P = 0.007 \); Fig. 4C).

Treatment response after TACE and radiotherapy according to EpCAM mRNA+ CTC level

Twenty patients receiving TACE and 22 patients receiving radiotherapy were enrolled to evaluate the treatment response using the optimized platform for CTC detection. Blood samples were collected pretreatment and 1 month after treatment and evaluated using the optimized qRT-PCR platform. MRI or CT scans were performed at the same time points, and mRECIST criteria were set as described in a previous report (30). The dynamic changes in CTC levels and the imaging changes of tumors were compared to investigate the value of the optimized platform for CTC detection in evaluation of treatment response. Compared with pretreatment samples, the proportion of EpCAM mRNA+ CTCs decreased significantly after TACE (relative EpCAM expression: 0–7.6 for pretreatment, 0–2.8 for posttreatment; 50.0% vs. 10.0%, \( P = 0.030 \); Fig. 4D), whereas no significant reduction in EpCAM mRNA+ CTCs was observed in the radiotherapy group (relative EpCAM expression: 0–15.2 for pretreatment, 0–22.5 for posttreatment; 54.5% vs. 45.4%, \( P = 0.700 \); Fig. 4D).

Next, we classified CTC dynamic changes pattern as “CTC elevation” and “CTC reduction” by direct comparison between pre- and posttreatment CTC levels. In the TACE group, none of the patients demonstrated CTC elevation, 10 (50.0%) patients showed CTC reduction and 10 (50.0%) patients remained CTC-negative. All the patients with CTC reduction showed tumor remission or stable disease according to the mRECIST criteria, and only one patient in the continuous negative CTC group (1/10) suffered tumor progression. In the radiotherapy group, 6 (27.3%) patients showed CTC elevation, 10 (45.4%) patients had CTC reduction, and 6 (27.3%) patients retained CTC-negative status. Four of 6 (66.7%) patients with CTC elevation suffered tumor progression according to the mRECIST criteria. None of the patients with CTC reduction and negative CTC status exhibited tumor progression. When we considered the TACE and radiotherapy data together, evaluation of treatment response by our CTC criteria was consistent with mRECIST criteria in 92.9% (39/42) of the paired samples (Table 2; \( r = 0.69, P < 0.010 \)).

Discussion

Measurement of CTCs in peripheral blood may serve as a surrogate diagnostic test that could constitute a “liquid biopsy” and provide real-time information about the patient’s current disease state (35, 36). In addition to their diagnostic and prognostic utility, CTCs are also an attractive alternative to tumor tissue for molecular characterization, which could potentially be used to select appropriate targeted therapy (21, 37, 38). Moreover, CTCs can easily be obtained from a routine blood draw with minimal risk to the patient.

In the present study, we constructed an optimized platform for CTC detection based on negative enrichment and qRT-PCR assay. Our platform showed a high specificity and sensitivity for detecting rare tumor cells in blood. To our knowledge, this is the first study to comprehensively evaluate the clinical significance of EpCAM mRNA+ CTCs in patients with HCC undergoing resection, TACE, or radiotherapy. Patients with pretreatment EpCAM mRNA+ CTCs had a significantly higher recurrence rate or worse PFS than patients without detectable CTCs. Moreover, dynamic changes in CTC levels detected by our qRT-PCR platform reflect the antitumor response, showing a high consistency with the mRECIST criteria. Thus, the detection of EpCAM mRNA+ CTC by our qRT-PCR platform might serve as a novel indicator for the risk of recurrence or progression in patients with HCC and a promising surrogate tool for surveillance of the therapeutic response instead of radiologic scans with the benefits of increased convenience and safety.

qRT-PCR is currently the most sensitive, rapid, and cost-effective technique for molecular identification of CTCs (20, 39, 40). The major drawback of this technique is the relatively high false-positive rate due to contamination with leukocytes, therefore pre-enrichment of CTCs is necessary (41). Consistent with early studies, our data showed that the false-positive rate was significantly increased without negative selection as a result of leukocyte contamination (Fig. 2A). After comparative testing, the RosetteSep Kit was finally adopted in our study based on the advantages of easy handling, high reproducibility, and high recovery rate. As the CTC subpopulation exhibits heterogeneity (25, 42), a negative enrichment method seems to be a better choice than positive enrichment targeting a specific CTC biomarker. It is rational to combine negative enrichment and qRT-PCR assays to decrease the false-positive rates and minimize the loss of CTCs as a result of heterogeneity of surface biomarkers. Using this strategy, our platform showed a high reproducibility (total coefficient variance: 2.97% for qRT-PCR assays to decrease the false-positive rates and minimize the loss of CTCs as a result of heterogeneity of surface biomarkers. Using this strategy, our platform showed a high reproducibility (total coefficient variance: 2.97% for Supplementary Table S3), and a 76.7% accordance between the qRT-PCR platform (5 mL blood sample) and the CellSearch system (7.5 mL blood sample) in clinical sample detection (Fig. 2D). Moreover, our data
also demonstrated the clinical significance of EpCAM mRNA-positive CTC detection by our qRT-PCR platform for predicting early recurrence and for surveillance of treatment efficiency in patients with HCC treated with surgery (Fig. 3C and D), similar to our previous studies based on the CellSearch system (14). Together, these data indicated that our platform could substitute for the CellSearch system for CTC detection with merits as follows: marker-independent enrichment manner for detecting heterogeneous CTC subpopulations, great potential in further molecule characterization, lower expense, and smaller blood volume requirement. More importantly, we believed that further molecular characterization of CTCs in patients with HCC using our qRT-PCR platform might result in more effective evaluation of the clinical significance of CTCs in risk prediction and treatment monitoring.

Using the single marker of EpCAM, our platform showed a moderate diagnostic performance in discrimination between patients with HCC, healthy volunteers, and patients without HCC with an AUC-ROC of 0.697 and a sensitivity of 42.6% and specificity of 96.7%. When combined with AFP, the AUC-ROC improved to 0.857, implying that the CTC test could enhance the discrimination capability of serum AFP and might be an effective supplement to serum AFP detection for HCC diagnosis (Fig. 3B). EpCAM mRNA-positive CTCs also showed diagnostic value in HCC subgroups including AFP < 20 ng/mL and early HCC (Supplementary Tables S3 and S4). Thus, although the diagnostic performance of the single marker was unsatisfactory for clinical use, a multimarker strategy with improved sensitivity and specificity might be qualified for clinical HCC diagnosis.

Our previous study reported that preoperative detection of EpCAM+ CTCs was an independent prognostic indicator for early recurrence in BCLC early-stage patients (14). However, the potential of EpCAM+ CTC detection as a prognostic indicator and for surveillance of treatment efficiency in patients with advanced HCC has not been reported. Similar to our previous study (14), Kaplan–Meier analysis demonstrated that a high pretreatment level of EpCAM mRNA-positive CTCs indicated a worse clinical outcome in patients with advanced BCLC stage regardless of treatment type (Fig. 4C). Moreover, our data showed that most of the patients with an elevated CTC level at the time of disease reassessment by imaging showed disease progression after TACE or radiotherapy, whereas patients with stable or decreasing CTC levels showed tumor remission or stable disease (Table 2). These data implied that CTC detection has

### Table 2. Correlation between CTC dynamic changes and imaging evaluation

<table>
<thead>
<tr>
<th>TACE</th>
<th>Radiotherapy</th>
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</thead>
<tbody>
<tr>
<td>CTC (2^\Delta C_q)</td>
<td>Treatment response</td>
</tr>
<tr>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>0</td>
<td>0.08</td>
</tr>
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<td>0</td>
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</tr>
<tr>
<td>0.73</td>
<td>0.75</td>
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<td>1.23</td>
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</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.37</td>
<td>0.2</td>
</tr>
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<td>0</td>
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</tr>
<tr>
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</tr>
<tr>
<td>4.82</td>
<td>1.58</td>
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<tr>
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<tr>
<td>2.38</td>
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<tr>
<td>5.46</td>
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</table>

Abbreviations: CR, complete regression; PD, progression disease; PR, partial regression; SD, stable disease.
a higher predictive value than traditional measures and might be an ideal substitute for radiologic modalities without the side effects associated with X-ray exposure or allergies to radiography agents.

The current study has the limitations of a small cohort size, short follow-up time, and data from a single study center. In addition, we did not enroll the small number of patients receiving sorafenib treatment in the present study. A prospective, multicenter, and comprehensive randomized clinical trial to validate the clinical significance of our qRT-PCR-based CTC detection platform in patients with HCC is currently in progress in our laboratory.

In this study, we established a novel optimized negative enrichment and qRT-PCR-based CTC detection platform for patients with HCC that exhibits high sensitivity, specificity, and reproducibility with a small sample volume requirement. Our results indicate that CTC detection by this platform might be clinically useful in auxiliary diagnosis, assessing the treatment response, and early decision-making to tailor the most effective antitumor strategies. More importantly, CTC molecular characterization based on this platform might play an important role in personalized therapy for patients with HCC in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Writing, review, and/or revision of the manuscript: W. Gao, X.-R. Yang, Y. Sun, M. Shen, X. Ma, C. Zhang, J. Wu, Y. Zhou, J. Fan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Hu, J. Fan
Figure designing: Y. Xu

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Clinical Significance of EpCAM mRNA-Positive CTCs in HCC


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Wei Guo, Xin-Rong Yang, Yun-Fan Sun, et al.


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