Plasma circular RNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma: A large-scale, multicenter study

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To explore whether plasma circular RNAs (circRNAs) can diagnose hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC), microarray and qPCR were used to identify plasma circRNAs that were increased in HBV-related HCC patients compared to controls (including healthy controls, chronic hepatitis B and HBV-related liver cirrhosis). A logistic regression model was constructed using a training set (n = 313) and then validated using another two independent sets (n = 306 and 526, respectively). Area under the receiver operating characteristic curve (AUC) was used to evaluate diagnostic accuracy. We identified a plasma circRNA panel (CircPanel) containing three circRNAs (hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897); circRNA: circular RNA; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; PCR: polymerase chain reaction; qRT-PCR: quantitative reverse transcription polymerase chain reaction; ROC: receiver operating characteristics

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Additional Supporting Information may be found in the online version of this article.

Key words: circular RNA, CircPanel, diagnosis, hepatocellular carcinoma, plasma

Abbreviations: AFP: alpha-fetoprotein; ALT: alanine aminotransferase; ANL: adjacent noncancerous liver; AUC: area under the receiver operating characteristic curve; BCLC: Barcelona Clinic Liver Cancer; CHB: chronic hepatitis B; CI: confidence interval; CircPanel: circRNA panel containing three circRNAs (hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897); circRNA: circular RNA; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; PCR: polymerase chain reaction; qRT-PCR: quantitative reverse transcription polymerase chain reaction; ROC: receiver operating characteristics

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hsa_circ_0139897) that could detect HCC. CircPanel showed a higher accuracy than AFP (alpha-fetoprotein) to distinguish individuals with HCC from controls in all three sets (AUC, 0.863 [95% confidence interval, CI: 0.819–0.907] vs. 0.790 [0.738–0.842], p = 0.036 in training set; 0.843 [0.796–0.890] vs. 0.747 [0.691–0.804], p = 0.011 in validation set 1 and 0.864 [0.830–0.898] vs. 0.769 [0.728–0.810], p < 0.001 in validation set 2). CircPanel also performed well in detecting Small-HCC (solitary, ≤ 3 cm), AFP-negative HCC and AFP-negative Small-HCC. Altogether, the findings point to CircPanel as a promising potential biomarker in HCC diagnosis.

Introduction
Hepatocellular carcinoma (HCC), largely attributable to chronic hepatitis B virus (HBV) infection, is the second most common gastrointestinal solid tumors and remains the second leading cause of cancer-related death in China.1 The high mortality of HCC is due partly to the fact that early-stage HCC shows no obvious symptoms and the diagnostic accuracy of AFP (alpha-fetoprotein), a serum biomarker for the diagnosis of HCC in clinical use) and other potential serum biomarkers (such as DCP [des-gamma-carboxyprothrombin], GPC3 [glypican-3], and microRNAs) is unsatisfactory. The sensitivities and specificities of high-serum AFP for HCC were reported to range from 39 to 64% and 76–91%, respectively.2–4 A meta-analysis showed that the sensitivity and specificity of DCP for the diagnosis of HCC were 71% and 84%, respectively.5 The sensitivities and specificities of GPC3 in diagnosing HCC ranging from 36 to 65% and 65–100%, respectively.6 A multicenter, retrospective study showed that the sensitivities and specificities of Cmi (the combination of seven serum microRNAs) in diagnosing HCC ranging from 70 to 86% and 80–91%, respectively.7 Therefore, a novel biomarker for the detection of HCC, especially early-stage HCC, need to be identified.

Circular RNAs (termed circRNAs) are covalently closed, single-stranded and stable transcripts.8 In our previous study, we demonstrated that circRNA expression profiles in HCC and adjacent nontumor liver tissues are significantly different and circular RNA cSMARCA5 (hsa_circ_0001445) inhibits the growth and metastasis of HCC.9 Serum/plasma circular RNAs can be biomarkers for the diagnosis of various cancers, including gastric cancer,10–12 breast cancer,13 lung cancer,14,15 pancreatic cancer16 and HCC.17,18 In particular, the sensitivity, specificity and area under the receiver operating characteristic curve (AUC) of serum circRNA_104075 for the diagnosis of HCC were 96%, 98% and 0.973, respectively.17 The AUCs of plasma cSMARCA5 in distinguishing HCC from healthy controls, hepatitis patients and liver cirrhosis patients were 0.938, 0.853 and 0.711, respectively. However, these two studies were single-center studies with limited participants.

In this study, using microarray and qRT-PCR (quantitative real-time polymerase chain reaction), we tried to explore whether plasma circRNAs can be biomarkers to diagnose HBV-related HCC (referred to below as HCC) in 1155 participants from three hospitals in China. They identified a plasma circRNA panel (CircPanel, including hsa_circ_0000976, hsa_circ_0007750, and hsa_circ_0139897) that showed higher accuracy than the clinically-used serum biomarker AFP in distinguishing individuals with HCC or Small-HCC from controls and performed well in diagnosing AFP-negative HCC and AFP-negative Small-HCC. Therefore, the findings point to CircPanel as a promising potential biomarker in HCC diagnosis.

What’s New?
To date, one limitation in the treatment of hepatocellular carcinoma (HCC) is the lack of serum biomarkers with satisfactory diagnostic accuracy. Here, the authors explored whether plasma circRNAs can be biomarkers to diagnose hepatitis B virus-related HCC in 1155 participants from three hospitals in China. They identified a plasma circRNA panel (CircPanel, including hsa_circ_0000976, hsa_circ_0007750, and hsa_circ_0139897) that showed higher accuracy than the clinically-used serum biomarker AFP in distinguishing individuals with HCC or Small-HCC from controls and performed well in diagnosing AFP-negative HCC and AFP-negative Small-HCC. Altogether, the findings point to CircPanel as a promising potential biomarker in HCC diagnosis.

Materials and Methods
Study design and participants
The study design is listed in Figure 1. In total, 1,195 plasma samples, 40 paired HCC and adjacent noncancerous liver (ANL) tissues were collected from three hospitals in China. The recruited participants were defined as healthy individuals, patients with chronic hepatitis B (CHB), patients with HBV-related liver cirrhosis (referred to below as liver cirrhosis) or patients with HCC by medical doctors, according to eligibility criteria listed in Table S1.

The plasma, HCC and ANL tissues in the discovery and training sets were collected between July 2016 and June 2017 at the Shanghai Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. The plasma in Validation Set 1 was collected between September 2016 and July 2017 at the Shanghai Changhai Hospital, Second Military Medical University, Shanghai, China. The plasma in Validation Set 2 was collected between February 2016 and May 2018 at the Mengchao Hepatobiliary Surgery Hospital, Fujian Medical University, Fuzhou, China. The details of the clinicopathological characteristics of the participants are listed in Table S2. In addition, for the 40 HCC patients undergoing hepatectomy in the training set, we also collected their plasma at the 30th day after hepatectomy, their HCC and paired ANL
The details of the clinicopathological characteristics of these 40 HCC patients are listed in Table S3.

Human specimen collection was approved by the ethics committee of each hospital. Written informed consent was obtained from each patient according to the policies of the committee.

HCC cell lines
The hepatoblastoma-derived Hep-G2 (RRID: CVCL_0027) and the HCC-derived Huh-7 (RRID: CVCL_0336) cell lines were obtained from the Chinese Academy of Sciences Cell Bank and were authenticated by short tandem repeat (STR) profiling within the last 3 years. All experiments were performed with mycoplasma-free cells. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (Gibco BRL). The cells were maintained in an atmosphere of 5% CO₂ in a humidified 37°C incubator.

RNA isolation
For the HCC cell lines and the HCC and ANL tissues, the total RNA was extracted using RNAiso Plus (Takara, Code No. 9109) according to the manufacturer’s instructions. For the plasma, the total RNA was extracted using the TRIzol™ LS Reagent (ThermoFisher, Code No. 10296010) according to the manufacturer’s instructions. During the isopropanol precipitation, glycogen (catalog number AM9510, Ambion/Applied Biosystems, Foster City, California) was added as a coprecipitant (final concentration of 100 μg/ml) to enhance the RNA precipitation.

CircRNA microarray expression profiling
The total RNAs extracted from the plasma of five HCC patients and five CHB patients were used for microarray analysis as described previously. In brief, the RNAs were digested, amplified, labeled and hybridized onto the microarray (CapitalBio Technology Human CircRNA Array, Version 2.0). Differential expression analysis of circRNAs was performed using GeneSpring software V13.0 (Agilent). We used threshold values of ≥2 or ≤2-fold change and a t-test p-value <0.05. The differentially expressed circRNAs are listed in Table S4. The data were log2 transformed and median centered by genes using the Adjust Data function of CLUSTER 3.0 software and were then further analyzed by hierarchical clustering with average linkage. Finally, we performed tree

Figure 1. Study design. There were 313 participants (53 healthy controls, 52 with CHB, 50 with liver cirrhosis and 158 with HCC) in the training set. For 40 HCC patients among them, we also collected their HCC tissues and ANL tissues during the surgery, and plasma samples 1 month after radical hepatectomy. Therefore, there were 353 plasma samples in the training set.
visualization by using Java Treeview (Stanford University School of Medicine, Stanford, California).

Reverse transcription
Total RNA from both tissues or plasma was reversely transcribed using M-MLV Reverse Transcriptase Kit (ThermoFisher, Code No. 28025021) according to the manufacturer's instructions.

Quantitative reverse-transcriptase polymerase chain reaction
The qRT-PCR, using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) and ROX plus (Takara, Code No. RR82LR), was performed on the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, California). The PCR primers for β-actin and the four candidate circRNAs (hsa_circ_0000976, hsa_circ_0003506, hsa_circ_0007750 and hsa_circ_0139897) are listed in Table S5. The primers for the circRNAs were divergent and circRNA specific (Fig. S1A).

For the HCC and ANL tissues, ACTB was employed as the endogenous control, and the relative expression was calculated using the comparative ΔΔCt method.

Because there is no accepted endogenous control for the quantitation of mRNAs/circRNAs in plasma, we used absolute quantitation when detecting the expression of the candidate circRNAs in plasma, as previously described.22–24 Briefly, the PCR products were amplified from human pooled plasma cDNA using the primers of the four candidate circRNAs, respectively. Subsequently, the four PCR products were cloned into pUC57 vector, respectively. The resulting constructs were sequenced and serially diluted from 5 × 10⁷ copies per microliter to 5 copies per microliter, respectively. Those diluted constructs were run in parallel with the samples under identical qPCR conditions and amplified with the same set of primers. A standard curve was generated by plotting the cycle threshold as a function of log₁₀ concentration of the serial diluted controls (Fig. S1B-S1E). The relative amount of cDNA of a particular template was extrapolated from the standard curve using the LightCycler software 3.0 (Bio-Rad).

Statistical analysis
All statistical analyses were performed using SPSS version 23.0 software (SPSS, Inc., Chicago, IL). For comparisons, chi-squared test, Student’s t test, Wilcoxon signed-rank test, Mann–Whitney U test and Kruskal–Wallis H test were performed, as appropriate. Correlations were measured by Spearman correlation analysis. The optimal cutoff values of the expression of the candidate circRNAs in plasma were determined by a ROC curve (Euclidean distance) analysis in Cutoff Finder25 (http://molpath.charite.de/cutoff/). Binary logistic regression was used to build the diagnostic model CircPanel (circRNA panel, including hsa_circ_0000976, hsa_circ_0003506 and hsa_circ_0139897) as described previously.23,26 Area under the receiver operating characteristic curve (AUC) was used to evaluate diagnostic accuracy. The comparison of AUC was performed by the pROC package of R software (version 3.0.1).7,27 All p values were two-sided. It was considered to be statistically significant when p < 0.05.

Data availability
The data that support the findings of this study are openly available in Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135806.

The remaining methods are described in Data S1.

Results
Identification of circular RNAs by microarray and PCR in human plasma samples
Using circRNA microarray, we compared the expression of circular RNAs in the plasma from five HCC patients and five CHB patients (discovery set). Among the 371 differentially expressed circRNAs, 326 were upregulated and 45 were downregulated in the plasma from HCC patients compared to that from CHB patients (Table S4). We hypothesized that circRNAs in HCC tissues could be secreted into plasma. By overlapping the 326 upregulated plasma circRNAs and the 6,584 circRNAs detected in HCC tissues from our previous study,9 we obtained 10 candidate circRNAs (Table S4). Subsequently, we successfully validated four of these (hsa_circ_0000976, hsa_circ_0003506, hsa_circ_0007750 and hsa_circ_0139897) in human plasma (Fig. S2B-S2D), human HCC tissues (Fig. S3A-S3C) and human HCC cell lines (Hep-G2 and Huh-7) (Fig. S4) and by qRT-PCR using circRNA-specific divergent primers, agarose gel electrophoresis and Sanger sequencing. Furthermore, we found that incubating plasma at room temperature for up to 24 hr had minimal effect on the expression of these four circRNAs (Fig. S2E), indicating that they were stable in plasma and could be used as a biomarker. In addition, after the treatment with RNase R (a highly processive 3′-to-5′ exoribonuclease that digests linear RNA28), none showed significant changes (Fig. S3D), which demonstrated that they were truly circular and not linear.

Subsequently, Hep-G2 and Huh-7 cells were subcutaneously implanted into the bilateral armpits of BALB/c nude mice. Twenty-eight days later (once tumors were well established), all mice were sacrificed, and their plasma was collected for the detection of the four candidate circRNAs. As expected, agarse gel electrophoresis following RT-PCR (Fig. S5) and Sanger sequencing (Fig. S6) showed that these four circRNAs existed in the plasma from the mice in the Hep-G2 and Huh-7 groups but not in the control group. This proved that HCC cells secrete circRNAs into plasma.

Building of the diagnostic model CircPanel based on the training set
By qRT-PCR, we detected the expression of hsa_circ_0000976, hsa_circ_0003506, hsa_circ_0007750 and hsa_circ_0139897 in the plasma from 158 HCC patients, 53 healthy controls, 52 CHB patients and 50 HBV-induced liver cirrhosis patients (Fig. 1) and
Table 1. The performance of CircPanel, AFP and their combination for the diagnosis of HCC

| Groups       | Training set | Validation Set 1 | Validation Set 2 |
|--------------|--------------|------------------|------------------|
|              | AUC (95% CI) | Sensitivity (%)  | Specificity (%)  | p value | AUC (95% CI) | Sensitivity (%)  | Specificity (%)  | p value |
| HCC vs. Non-HCC |              |                  |                  |         |              |                  |                  |         |
| CircPanel    | 0.861 (0.819–0.907) | 81.6             | 91.0             | 0.036#  | 0.843 (0.796–0.890) | 87.5             | 81.2             | 0.011#  |
|              |              |                  |                  |         |              |                  |                  |         | 0.864 (0.830–0.898) | 85.5             | 87.3     | **0.001#**  |
| AFP          | 0.790 (0.738–0.842) | 68.4             | 89.7             | 0.010$  | 0.747 (0.691–0.804) | 60.5             | 89.0             | 0.002$  |
|              |              |                  |                  |         |              |                  |                  |         | 0.769 (0.728–0.810) | 65.2             | 88.6     | **0.001$**  |
| CircPanel+AFP| 0.878 (0.836–0.920) | 91.8             | 83.9             | 0.622$  | 0.863 (0.819–0.908) | 92.8             | 79.9             | 0.547$  |
|              |              |                  |                  |         |              |                  |                  |         | 0.874 (0.840–0.907) | 91.7             | 83.1     | 0.685$      |
| HCC vs. Healthy |              |                  |                  |         |              |                  |                  |         |
| CircPanel    | 0.861 (0.803–0.919) | 81.6             | 90.6             | 0.632#  | 0.858 (0.791–0.924) | 87.5             | 84.0             | 0.158#  |
|              |              |                  |                  |         |              |                  |                  |         | 0.875 (0.829–0.921) | 85.5             | 89.5     | **0.084#**  |
| AFP          | 0.842 (0.791–0.893) | 100.0            | 100.0            | 0.057$  | 0.793 (0.731–0.854) | 60.5             | 98.0             | **0.044$**  |
|              |              |                  |                  |         |              |                  |                  |         | 0.819 (0.777–0.862) | 65.2             | 98.7     | **0.006$**  |
| CircPanel+AFP| 0.912 (0.860–0.964) | 91.8             | 90.6             | 0.199$  | 0.884 (0.820–0.948) | 92.8             | 84.0             | 0.583$  |
|              |              |                  |                  |         |              |                  |                  |         | 0.906 (0.862–0.950) | 91.7             | 89.5     | 0.341$      |
| HCC vs. CHB  |              |                  |                  |         |              |                  |                  |         |
| CircPanel    | 0.870 (0.814–0.925) | 81.6             | 92.3             | 0.024#  | 0.817 (0.744–0.891) | 87.5             | 75.9             | 0.097#  |
|              |              |                  |                  |         |              |                  |                  |         | 0.859 (0.809–0.908) | 85.5             | 86.3     | **0.001#**  |
| AFP          | 0.766 (0.693–0.837) | 84.6             | 84.6             | 0.031$  | 0.729 (0.654–0.803) | 60.5             | 85.2             | 0.051$  |
|              |              |                  |                  |         |              |                  |                  |         | 0.732 (0.672–0.792) | 65.2             | 81.3     | **0.007$**  |
| CircPanel+AFP| 0.872 (0.807–0.937) | 91.8             | 82.7             | 0.963$  | 0.834 (0.761–0.908) | 92.8             | 74.1             | 0.749$  |
|              |              |                  |                  |         |              |                  |                  |         | 0.846 (0.789–0.903) | 91.7             | 77.5     | 0.741$      |
| HCC vs. Cirrhosis |          |                  |                  |         |              |                  |                  |         |
| CircPanel    | 0.858 (0.798–0.918) | 81.6             | 90.0             | 0.047#  | 0.858 (0.791–0.924) | 87.5             | 84.0             | 0.009#  |
|              |              |                  |                  |         |              |                  |                  |         | 0.859 (0.809–0.908) | 85.5             | 86.3     | **0.008#**  |
| AFP          | 0.762 (0.688–0.835) | 84.0             | 84.0             | 0.098$  | 0.723 (0.645–0.800) | 60.5             | 84.0             | **0.004$**  |
|              |              |                  |                  |         |              |                  |                  |         | 0.757 (0.701–0.813) | 65.2             | 86.3     | **0.003$**  |
| CircPanel+AFP| 0.840 (0.777–0.921) | 91.8             | 78.0             | 0.845$  | 0.874 (0.807–0.941) | 92.8             | 82.0             | 0.739$  |
|              |              |                  |                  |         |              |                  |                  |         | 0.871 (0.819–0.923) | 91.7             | 82.5     | 0.737$      |

The p values indicate the statistical significance for the differences of AUC between CircPanel and AFP (#), CircPanel+AFP and AFP ($) or CircPanel+AFP and CircPanel (§). It is considered to be statistically significant when \( p < 0.05 \) (in bold).

Abbreviations: AFP, alpha fetoprotein; CHB, chronic hepatitis B; CircPanel, circRNA panel containing three circRNAs (hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897); HCC, hepatocellular carcinoma.
found that the expression of hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897 (but not hsa_circ_0003506) in the plasma from the HCC patients was higher than that in the plasma from the healthy controls, CHB patients and liver cirrhosis patients (Fig. S7A). For the 40 HCC patients undergoing heptectomy in the training set, we also detected the expression of these four circRNAs in their plasma at the 30th day after heptectomy and in their HCC and paired ANL tissues. We found that the expression of hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897 (but not hsa_circ_0003506) in the plasma from the HCC patients was positively correlated with the expression in their HCC tissues (Fig. S7B) and was significantly downregulated after heptectomy (Fig. S7C). Furthermore, the expression of hsa_circ_0000976 and hsa_circ_0007750 was higher in the HCC tissues than in the ANL tissues (Fig. S7D). Therefore, we chose hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897 as the candidate circRNAs for the diagnosis of HCC.

Using Cutoff Finder25 (http://molpath.charite.de/cutoff/), we determined that the best cutoff values of plasma hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897 were 1,067, 4,324 and 1,108 copies per milliliter of plasma, respectively. Their diagnostic performance is shown in Table S6.

To improve diagnostic accuracy, using binary logistic regression, we built four diagnostic models by combining all the three circRNAs (hsa_circ_0000976 + hsa_circ_0007750 + hsa_circ_0139897, CircPanel) or two of them (hsa_circ_0000976 + hsa_circ_0007750, hsa_circ_0000976 + hsa_circ_0139897 or hsa_circ_0007750 + hsa_circ_0139897). The predicted probability of being detected as HCC by the CircPanel was calculated by Logit \(\text{p} = -3.502 + 1.920 \times \text{hsa}_c_{\text{irc}}_0000976 + 2.800 \times \text{hsa}_c_{\text{irc}}_0007750 + 3.154 \times \text{hsa}_c_{\text{irc}}_0139897\). In this equation, the circRNA symbol was substituted with the discretized value 1 when the level of the circRNA was higher than the corresponding best cutoff value; otherwise, it was substituted with the discretized value of zero. If the result of \(\text{logit(p} = \text{HCC})\) was higher than 0.5, then the detected sample was predicted as HCC; otherwise, it was Non-HCC (Table S6).

Similarly, the predicted probability of being detected as HCC by two circRNAs was also calculated (Table S6).

As a result, the diagnostic accuracy of the CircPanel was higher than that of either single circRNA or the combination with either two circRNAs (Table S6). Therefore, CircPanel was chosen for further study.

Figure 2. The performance of CircPanel, AFP and their combination for the diagnosis of HCC in the training set (a–d), Validation Set 1 (e–h) and Validation Set 2 (i–l). Their detailed diagnostic performance was listed in Table 1. [Color figure can be viewed at wileyonlinelibrary.com]
Table 2. The performance of CircPanel, AFP and their combination for the diagnosis of Small-HCC

| Groups                  | Training set |       |       |       | Validation Set 1 |       |       |       | Validation Set 2 |       |       |       |
|-------------------------|--------------|-------|-------|-------|------------------|-------|-------|-------|------------------|-------|-------|-------|
|                         | AUC (95% CI) | Sensitivity (%) | Specificity (%) | p value | AUC (95% CI) | Sensitivity (%) | Specificity (%) | p value | AUC (95% CI) | Sensitivity (%) | Specificity (%) | p value |
| Small-HCC vs. Non-HCC   |              |       |       |       |                  |       |       |       |                  |       |       |       |
| CircPanel               | 0.862 (0.796–0.928) | 81.5 | 91.0 | 0.001# | 0.838 (0.776–0.900) | 86.4 | 81.2 | 0.011# | 0.851 (0.799–0.903) | 83.0 | 87.3 | 0.009# |
| AFP                     | 0.680 (0.589–0.770) | 46.3 | 89.7 | 0.001$ | 0.699 (0.613–0.785) | 50.8 | 89.0 | 0.001$ | 0.738 (0.671–0.805) | 59.1 | 88.6 | 0.002$ |
| CircPanel+AFP           | 0.873 (0.817–0.929) | 90.7 | 83.9 | 0.809§ | 0.874 (0.823–0.925) | 94.9 | 79.9 | 0.383§ | 0.864 (0.818–0.910) | 89.8 | 83.1 | 0.715§ |
| Small-HCC vs. Healthy   |              |       |       |       |                  |       |       |       |                  |       |       |       |
| CircPanel               | 0.860 (0.784–0.936) | 81.5 | 90.6 | 0.041# | 0.852 (0.774–0.930) | 86.4 | 84.0 | 0.080# | 0.862 (0.801–0.923) | 83.0 | 89.5 | 0.124# |
| AFP                     | 0.731 (0.634–0.829) | 46.3 | 100.0 | 0.003$ | 0.744 (0.651–0.837) | 50.8 | 98.0 | 0.010$ | 0.789 (0.718–0.860) | 59.1 | 98.7 | 0.018$ |
| CircPanel+AFP           | 0.907 (0.843–0.971) | 90.7 | 90.6 | 0.362§ | 0.895 (0.826–0.963) | 94.9 | 84.0 | 0.419§ | 0.896 (0.842–0.950) | 89.8 | 89.5 | 0.412§ |
| Small-HCC vs. CHB       |              |       |       |       |                  |       |       |       |                  |       |       |       |
| CircPanel               | 0.869 (0.795–0.943) | 81.5 | 92.3 | 0.001# | 0.812 (0.728–0.896) | 86.4 | 75.9 | 0.048# | 0.846 (0.783–0.909) | 83.0 | 86.3 | 0.005# |
| AFP                     | 0.655 (0.550–0.759) | 46.3 | 84.6 | 0.001$ | 0.680 (0.581–0.779) | 50.8 | 85.2 | 0.011$ | 0.702 (0.622–0.782) | 59.1 | 81.3 | 0.010$ |
| CircPanel+AFP           | 0.867 (0.792–0.942) | 90.7 | 82.7 | 0.974§ | 0.845 (0.767–0.923) | 94.9 | 74.1 | 0.574§ | 0.836 (0.771–0.902) | 89.8 | 77.5 | 0.835§ |
| Small-HCC vs. Cirrhosis |              |       |       |       |                  |       |       |       |                  |       |       |       |
| CircPanel               | 0.857 (0.780–0.935) | 81.5 | 90.0 | 0.002# | 0.852 (0.774–0.930) | 86.4 | 84.0 | 0.007# | 0.846 (0.783–0.909) | 83.0 | 86.3 | 0.019# |
| AFP                     | 0.651 (0.546–0.757) | 46.3 | 84.0 | 0.005$ | 0.674 (0.573–0.776) | 50.8 | 86.0 | 0.001$ | 0.727 (0.649–0.804) | 59.1 | 86.3 | 0.007$ |
| CircPanel+AFP           | 0.844 (0.762–0.925) | 90.7 | 78.0 | 0.811§ | 0.885 (0.813–0.956) | 94.9 | 82.0 | 0.540§ | 0.861 (0.800–0.922) | 89.8 | 82.5 | 0.732§ |

The p values indicate the statistical significance for the differences of AUC between CircPanel and AFP (#), CircPanel+AFP and AFP ($) or CircPanel+AFP and CircPanel (§). It is considered to be statistically significant when p < 0.05 (in bold).

Abbreviations: AFP, alpha fetoprotein; CHB, chronic hepatitis B; CircPanel, circRNA panel containing three circRNAs (hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897); HCC, hepatocellular carcinoma; Small-HCC, solitary, diameter ≤ 3 cm.
We also analyzed the diagnostic performance of AFP, whose recommended clinical cutoff is 20 ng/ml, in detecting HCC in the training set (Table 1). Furthermore, using the aforementioned method, we combined the CircPanel and AFP (CircPanel+AFP) to diagnosis HCC. The predicted probability of being detected as HCC by CircPanel+AFP was calculated as Logit ($p=\text{HCC}$) = $-2.152 + 3.321 \times \text{CircPanel} + 2.241 \times \text{AFP}$. In this equation, the CircPanel symbol was substituted with the discretized value 1 when the detected sample was diagnosed as HCC by CircPanel alone; otherwise, it was substituted with the discretized value of zero. The AFP symbol was substituted with the discretized value 1 when the serum AFP level was higher than 20 ng/ml; otherwise, it was substituted with the discretized value of zero. The diagnostic performance of CircPanel+AFP was then analyzed (Table 1).

**Performance of CircPanel, AFP and their combination for the diagnosis of HCC**

We then detected the expression of hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897 in the plasma from Validation Set 1 (152 HCC patients, 50 healthy controls, 54 CHB patients and 50 HBV-induced liver cirrhosis patients) and Validation Set 2 (290 HCC patients, 76 healthy controls, 80 CHB patients and 80 HBV-induced liver cirrhosis patients) and analyzed the performance of the CircPanel, AFP and their combination (CircPanel+AFP) for the diagnosis of HCC.

As a result, we found that both the CircPanel and CircPanel+AFP showed a higher accuracy than AFP in distinguishing individuals with HCC from Non-HCC in all three sets (CircPanel vs. AFP: AUC 0.863 [95% confidence interval, CI: 0.819–0.907] vs. 0.790 [0.738–0.842], $p = 0.036$ in the training set; 0.843 [0.796–0.890] vs. 0.747 [0.691–0.804], $p = 0.011$ in Validation Set 1 and 0.864 [0.830–0.898] vs. 0.769 [0.728–0.810], $p < 0.001$ in Validation Set 2. CircPanel+AFP vs. AFP: 0.878 [0.836–0.920] vs. 0.790 [0.738–0.842], $p = 0.010$ in the training set; 0.863 [0.819–0.908] vs. 0.747 [0.691–0.804], $p = 0.002$ in Validation Set 1 and 0.874 [0.840–0.907] vs. 0.769 [0.728–0.810], $p < 0.001$ in Validation Set 2). In addition, the CircPanel and CircPanel+AFP were not significantly different in distinguishing HCC from Non-HCC (Table 1, Fig. 2).

Subsequently, we divided the Non-HCC group into healthy, CHB and liver cirrhosis groups and analyzed the diagnostic performance of CircPanel, AFP and CircPanel+AFP in HCC vs. Healthy, HCC vs. CHB and HCC vs. Cirrhosis. The results were similar, especially for HCC vs. CHB and HCC vs. cirrhosis (Table 1, Fig. 2).

![Figure 3](image-url) The performance of CircPanel, AFP and their combination for the diagnosis of Small-HCC in the training set (a–d), Validation Set 1 (e–h) and Validation Set 2 (i–l). Their detailed diagnostic performance was listed in Table 2. Small-HCC, solitary, diameter ≤3 cm. [Color figure can be viewed at wileyonlinelibrary.com]
Plasma circular RNA to diagnose HCC

Performance of CircPanel, AFP and their combination for the diagnosis of Small-HCC

We then analyzed the performance of CircPanel, AFP and their combination (CircPanel+AFP) in the diagnosis of Small-HCC (solitary, diameter ≤ 3 cm) and found that both the CircPanel and CircPanel+AFP showed a higher accuracy than AFP in distinguishing individuals with Small-HCC from Non-HCC in all three sets (CircPanel vs. AFP: 0.881 [0.797–0.965] vs. 0.680 [0.589–0.770], \( p = 0.001 \) in the training set; 0.878 [0.785–0.971] vs. 0.699 [0.613–0.785], \( p = 0.011 \) in Validation Set 1 and 0.851 [0.799–0.903] vs. 0.738 [0.671–0.805], \( p = 0.009 \) in Validation Set 2). In addition, the CircPanel and CircPanel+AFP did not show a significant difference in distinguishing Small-HCC from Non-HCC (Table 2, Fig. 3).

Subsequently, we divided the Non-HCC group into healthy, CHB and liver cirrhosis groups and analyzed the diagnostic performance of the CircPanel, AFP and CircPanel+AFP in Small-HCC vs. Healthy, Small-HCC vs. CHB and Small-HCC vs. Cirrhosis. Similar results were obtained, especially in Small-HCC vs. CHB and Small-HCC vs. cirrhosis (Table 2, Fig. 3).

Performance of CircPanel for the diagnosis of AFP-negative HCC and AFP-negative Small-HCC

Furthermore, we analyzed the performance of the CircPanel in the diagnosis of AFP-negative HCC and AFP-negative Small-HCC. The results showed that the CircPanel also had a high diagnostic accuracy (all AUCs were higher than 0.800; Table 3).

Discussion

Our study is unique for the following reasons. First, to our knowledge, this is the first report to compare the expression of circRNAs in the plasma from HCC and CHB patients by microarray. Furthermore, it was a multicenter study with 1,155 participants. Importantly, the three circRNAs in the CircPanel proved to be positively correlated with that in HCC tissues, though the correlation coefficient was relatively low. In addition, the CircPanel showed higher accuracy than AFP in distinguishing individuals with HCC or Small-HCC from the controls and performed well in diagnosing AFP-negative HCC and AFP-negative Small-HCC. All these findings make the CircPanel a compelling diagnostic biomarker.

There are a few limitations in the present study. First, all the HCC patients in this study were HBV-related. Further studies are needed to evaluate the performance of the CircPanel in diagnosing HCC caused by other factors. Second, although the expression of hsa_circ_0139897 in HCC and ANL tissues did not show a significant difference, its expression in the plasma from HCC patients was positively correlated with that in their HCC tissues and was significantly downregulated after heptectomy. The reason for this discrepancy is not clear at the present time and needs further

Table 3. The performance of CircPanel for the diagnosis of AFP-negative HCC and AFP-negative Small-HCC

| Groups                | Training set | Validation Set 1 | Validation Set 2 |
|-----------------------|--------------|------------------|------------------|
|                       | AUC (95% CI) | Sensitivity (%)  | Specificity (%)  |
|                       |              |                  |                  |
| HCC vs. Non-HCC       | 0.838 (0.769–0.909) | 88.8             | 95.5             |
| HCC vs. Healthy       | 0.849 (0.779–0.939) | 88.7             | 96.6             |
| HCC vs. CHB           | 0.894 (0.824–0.961) | 88.7             | 97.7             |
| HCC vs. Cirrhosis     | 0.881 (0.817–0.946) | 88.7             | 97.6             |
| Small-HCC vs. Healthy | 0.878 (0.806–0.949) | 89.7             | 97.0             |
| Small-HCC vs. CHB     | 0.892 (0.827–0.961) | 89.7             | 97.6             |
| Small-HCC vs. Cirrhosis | 0.887 (0.814–0.955) | 89.7             | 97.5             |

Abbreviations: AFP, alpha fetoprotein; CHB, chronic hepatitis B; CircPanel, circRNA panel containing three circRNAs (hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897); HCC, hepatocellular carcinoma; Small-HCC, solitary, diameter ≤ 3 cm.

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expansion. One possible explanation is that HCC tissues may secrete more hsa_circ_0139897 than ANL tissues. Third, a nested case–control study should be performed to evaluate the diagnostic performance of the plasma CircPanel in detecting preclinical HCC. Furthermore, the follow-up of the HCC patients should be continued in order to analyze the relationship between the plasma CircPanel and the prognosis of HCC patients. In addition, because the expression of hsa_circ_0000976 and hsa_circ_0007750 in the HCC tissues was higher than in the ANL tissues, their role in HCC progression awaits further investigation.

In summary, by a microarray screening and qRT-PCR in a multicenter study, we identified a plasma circRNA panel (CircPanel) containing three circRNAs (hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897) that detected HCC. The CircPanel performed better than AFP in diagnosing HCC and Small-HCC and also identified AFP-negative HCC and AFP-negative Small-HCC effectively. Therefore, we believe that the CircPanel can be a potential biomarker in the clinical diagnosis of HCC.

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