

1 Plasma Circular RNA Panel to Diagnose Hepatitis B Virus-Related Hepatocellular Carcinoma

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22 **Abbreviations:** AFP, alpha-fetoprotein; ALT, alanine aminotransferase; ANL, adjacent noncancerous liver;

23 AUC, area under the receiver operating characteristic curve; BCLC, Barcelona Clinic Liver Cancer; CHB,

1 chronic hepatitis B; CI, confidence interval; CircPanel, circRNA panel containing three circRNAs
2 (hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897); circRNA, circular RNA; HBsAg, hepatitis B
3 surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction;
4 qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROC, receiver operating
5 characteristics.
6

Abstract

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 HCC patients compared with controls (including healthy controls, chronic hepatitis B, HBV-related liver cirrhosis and HCC patients). 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) 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% 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.

Significance of this study

What is already known about this subject ?

1. The diagnostic accuracy of alpha-fetoprotein (AFP) in detecting hepatocellular carcinoma (HCC) is unsatisfactory.
2. Circular RNA (circRNA) expression profiles in HCC and adjacent nontumor liver tissues are significantly different.
3. Plasma circRNAs are enriched, stable and can be biomarkers for various diseases.

What are the new findings ?

1. The expression of circRNAs in the plasma from HCC patients and chronic hepatitis B is significantly different.
2. Plasma circRNA panel (CircPanel, including hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897) has a higher accuracy than AFP to distinguish individuals with HCC or Small-HCC (solitary, ≤ 3 cm) from controls (healthy controls, chronic hepatitis B and HBV-related liver cirrhosis).
3. CircPanel also performs well in diagnosing AFP-negative HCC and AFP-negative Small-HCC.

How might it impact on clinical practice in the foreseeable future?

Plasma CircPanel can be a diagnostic biomarker in detecting HCC and improves the diagnostic accuracy.

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 China¹. 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) is unsatisfactory. The sensitivity and specificity of high serum AFP for HCC were reported to range from 39–64% and 76–91%, respectively.²⁻⁴ 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.⁵ In our previous study, we demonstrated that circRNA expression profiles in HCC and adjacent nontumor liver tissues are significantly different and circular RNA cSMARCA5 inhibits the growth and metastasis of HCC.⁶ Furthermore, it has been reported that plasma circRNAs are enriched, stable and can be biomarkers for non-small cell lung cancer and systemic lupus erythematosus⁷. 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).

1 Patients and Methods

2 Study Design and Participants

3 The study design is listed in Figure 1. In total, 1195 plasma samples, 40 paired HCC and adjacent
4 noncancerous liver (ANL) tissues were collected from three hospitals in China. The recruited participants
5 were defined as healthy individuals, patients with chronic hepatitis B (CHB), patients with HBV-related
6 liver cirrhosis (referred to below as liver cirrhosis), or patients with HCC by medical doctors, according to
7 eligibility criteria listed in Supplementary Table 1.

8 The plasma, HCC and ANL tissues in the discovery and training sets were collected between July 2016
9 to June 2017 at the Shanghai Eastern Hepatobiliary Surgery Hospital, Second Military Medical University,
10 Shanghai, China. The plasma in validation set 1 was collected between September 2016 to July 2017 at the
11 Shanghai Changhai Hospital, Second Military Medical University, Shanghai, China. The plasma in
12 validation set 2 was collected between February 2016 to May 2018 at the Mengchao Hepatobiliary Surgery
13 Hospital, Fujian Medical University, Fuzhou, China. The details of the clinicopathological characteristics of
14 the participants are listed in Supplementary Table 2. In addition, for the 40 HCC patients undergoing
15 hepatectomy in the training set, we also collected their plasma at the 30th day after hepatectomy, their HCC
16 and paired ANL tissues. The details of the clinicopathological characteristics of these 40 HCC patients are
17 listed in Supplementary Table 3.

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

20 RNA Isolation

21 For the HCC cell lines and the HCC and ANL tissues, the total RNA was extracted using RNAiso Plus
22 (Takara, Code No. 9109) according to the manufacturer's instructions.

23 For the plasma, the total RNA was extracted using the TRIzol™ LS Reagent (ThermoFisher, Code No.

1 10296010) according to the manufacturer's instructions. During the isopropanol precipitation, glycogen
2 (catalogue number AM9510, Ambion/Applied Biosystems, Foster City, CA) was added as a coprecipitant
3 (final concentration of 100 µg/mL) to enhance the RNA precipitation.

4 **CircRNA Microarray Expression Profiling**

5 The total RNAs extracted from the plasma of five HCC patients and five CHB patients were used for
6 microarray analysis as described previously.^{8,9} In brief, the RNAs were digested, amplified, labelled and
7 hybridized onto the microarray (CapitalBio Technology Human CircRNA Array, Version 2.0). Differential
8 expression analysis of circRNAs was performed using GeneSpring software V13.0 (Agilent). We used
9 threshold values of ≥ 2 or ≤ -2 -fold change and a t-test P-value < 0.05 . The differentially expressed
10 circRNAs are listed in Supplementary Table 4. The data were Log₂ transformed and median centered by
11 genes using the Adjust Data function of CLUSTER 3.0 software and were then further analyzed by
12 hierarchical clustering with average linkage.¹⁰ Finally, we performed tree visualization by using Java
13 Treeview (Stanford University School of Medicine, Stanford, CA, USA).

14 **Reverse Transcription**

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

17 **Quantitative Reverse-transcriptase Polymerase Chain Reaction (qRT-PCR)**

18 The qRT-PCR, using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) and ROX plus (Takara, Code No.
19 RR82LR), was performed on the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City,
20 CA). The PCR primers for β -actin and the four candidate circRNAs (hsa_circ_0000976, hsa_circ_0003506,
21 hsa_circ_0007750 and hsa_circ_0139897) are listed in Supplementary Table 5. The primers for the
22 circRNAs were divergent and circRNA specific. (Supplementary Figure 1A)

23 For the HCC and ANL tissues, ACTB was employed as the endogenous control, and the relative

1 expression was calculated using the comparative $\Delta\Delta\text{Ct}$ method.

2 Since there is no accepted endogenous control for the quantitation of mRNAs/circRNAs in plasma, we
3 used absolute quantitation when detecting the expression of the candidate circRNAs in the plasma as
4 previously described.¹¹⁻¹³ Briefly, the PCR products were amplified from human pooled plasma cDNA using
5 the primers of the four candidate circRNAs, respectively. Subsequently, the four PCR products were cloned
6 into pUC57 vector, respectively. The resulting constructs were verified by direct sequencing and serially
7 diluted from 5×10^7 copies/ μl to 5 copies/ μl , respectively. Those diluted constructs were run in parallel with
8 the samples under identical qPCR conditions and amplified with the same set of primers. A standard curve
9 was generated by plotting the cycle threshold as a function of \log_{10} concentration of the serial diluted
10 controls (Supplementary Figure 1B-E). The relative amount of cDNA of a particular template was
11 extrapolated from the standard curve using the LightCycler software 3.0 (Bio-Rad).

12 **Statistical Analysis**

13 All statistical analyses were performed using SPSS version 23.0 software (SPSS, Inc., Chicago, IL).
14 For comparisons, chi-squared test, Student's t test, Wilcoxon signed-rank test, Mann-Whitney U test and
15 Kruskal-Wallis H test were performed, as appropriate. Correlations were measured by Spearman correlation
16 analysis. The optimal cut-off values of the expression of the candidate circRNAs in plasma were determined
17 by a ROC curve (Euclidean distance) analysis in Cutoff Finder¹⁴ (<http://molpath.charite.de/cutoff/>). Binary
18 logistic regression was used to build the diagnostic model CircPanel (circRNA panel, including
19 hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897) as described previously.^{12,15,16} Area under the
20 receiver operating characteristic curve (AUC) was used to evaluate diagnostic accuracy. The comparison of
21 AUC was performed by the pROC package of R software (version 3.0.1).^{15,17} All P values were two sided. It
22 was considered to be statistically significant when $P < 0.05$.

23 **The remaining methods are described in the Supplementary Data 1.**

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 with that from CHB patients (Supplementary Table 4, Supplementary Figure 2A). We hypothesized that circRNAs in HCC tissues could be secreted into plasma. By overlapping the 326 upregulated plasma circRNAs and the 6584 circRNAs detected in HCC tissues from our group's previous study⁶, we obtained 10 candidate circRNAs (Supplementary Table 4). Subsequently, we successfully validated four of these (hsa_circ_0000976, hsa_circ_0003506, hsa_circ_0007750 and hsa_circ_0139897) in human plasma (Supplementary Figure 2B-D), human HCC tissues (Supplementary Figure 3A-C), and human HCC cell lines (HepG2 and Huh7) (Supplementary Figure 4) 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 h had minimal effect on the expression of these four circRNAs (Supplementary Figure 2E), 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 RNAs¹⁸), none showed significant changes (Supplementary Figure 3D), which demonstrated that they were truly circular and not linear.

Subsequently, HepG2 and Huh7 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, agarose gel electrophoresis following RT-PCR (Supplementary Figure 5) and Sanger sequencing (Supplementary Figure 6) showed that these four circRNAs existed in the plasma from the mice in the HepG2 and Huh7 groups but

1 not in the control group. This proved that HCC cells secrete circRNAs into plasma.

2 **The Building of the Diagnostic Model CircPanel Based on the Training Set**

3 By qRT-PCR, we detected the expression of hsa_circ_0000976, hsa_circ_0003506, hsa_circ_0007750
4 and hsa_circ_0139897 in the plasma from 158 HCC patients, 53 healthy controls, 52 CHB patients and 50
5 HBV-induced liver cirrhosis patients (Figure 1) and found that the expression of hsa_circ_0000976,
6 hsa_circ_0007750 and hsa_circ_0139897 (but not hsa_circ_0003506) in the plasma from the HCC patients
7 was higher than that in the plasma from the healthy controls, CHB patients and liver cirrhosis patients
8 (Supplementary Figure 7A). For the 40 HCC patients undergoing hepatectomy in the training set, we also
9 detected the expression of these four circRNAs in their plasma at the 30th day after hepatectomy and in their
10 HCC and paired ANL tissues. We found that the expression of hsa_circ_0000976, hsa_circ_0007750 and
11 hsa_circ_0139897 (but not hsa_circ_0003506) in the plasma from the HCC patients was positively
12 correlated with the expression in their HCC tissues (Supplementary Figure 7B) and was significantly
13 downregulated after hepatectomy (Supplementary Figure 7C). Furthermore, the expression of
14 hsa_circ_0000976 and hsa_circ_0007750 was higher in the HCC tissues than in the ANL tissues
15 (Supplementary Figure 7D). Therefore, we chose hsa_circ_0000976, hsa_circ_0007750 and
16 hsa_circ_0139897 as the candidate circRNAs for the diagnosis of HCC.

17 Using Cutoff Finder¹⁴ (<http://molpath.charite.de/cutoff/>), we determined that the best cutoff values of
18 plasma hsa_circ_0000976, hsa_circ_0007750 and hsa_circ_0139897 for distinguishing HCC and Non-HCC
19 (healthy controls, CHB patients and liver cirrhosis patients as a whole) were 1067, 4324 and 1108 copies/ml
20 of plasma, respectively. Their diagnostic performance is shown in Supplementary Table 6. To improve
21 diagnostic accuracy, using binary logistic regression, we built the diagnostic model CircPanel. The predicted
22 probability of being detected as HCC by the CircPanel was calculated by: $\text{Logit}(P = \text{HCC}) =$
23 $-3.502 + 1.920 * \text{hsa_circ_0000976} + 2.800 * \text{hsa_circ_0007750} + 3.154 * \text{hsa_circ_0139897}$. In this equation, the

1 circRNA symbol was substituted with the discretized value one when the level of the circRNA was higher
2 than the corresponding best cutoff value; otherwise, it was substituted with the discretized value of zero. If
3 the result of $\text{logit}[p=\text{HCC}]$ was higher than 0.5, then the detected sample was predicted as HCC; otherwise it
4 was Non-HCC. As expected, the diagnostic accuracy of the CircPanel was higher than those of
5 hsa_circ_0000976 (AUC 0.863 [0.819–0.907] vs 0.702 [0.644–0.761], $P<0.001$), hsa_circ_0007750 (AUC
6 0.863 [0.819–0.907] vs 0.776 [0.723–0.830], $P=0.012$) and hsa_circ_0139897 (AUC 0.863 [0.819–0.907] vs
7 0.749 [0.693–0.804], $P=0.001$) (Supplementary Table 6).

8 We also analyzed the diagnostic performance of AFP, whose recommended clinical cutoff is 20 ng/ml,
9 in detecting HCC in the training set (Table 1). Furthermore, using the aforementioned method, we combined
10 the CircPanel and AFP (CircPanel+AFP) to diagnosis HCC. The predicted probability of being detected as
11 HCC by CircPanel+AFP was calculated as $\text{Logit}(P = \text{HCC}) = -2.152 + 3.321 * \text{CircPanel} + 2.241 * \text{AFP}$. The
12 diagnostic performance of CircPanel+AFP was then analyzed (Table 1).

13 **The Performance of CircPanel, AFP and Their Combination for the Diagnosis of HCC**

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

19 As a result, we found that both the CircPanel and CircPanel+AFP showed a higher accuracy than AFP
20 in distinguishing individuals with HCC from Non-HCC in all three sets (CircPanel versus AFP: AUC 0.863
21 [95% 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
22 [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
23 validation set 2. CircPanel+AFP versus AFP: 0.878 [0.836–0.920] vs 0.790 [0.738–0.842], $P=0.010$ in the

1 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–
2 0.907] vs 0.769 [0.728–0.810], P<0.001 in validation set 2). In addition, the CircPanel and CircPanel+AFP
3 were not significantly different in distinguishing HCC from Non-HCC (Table 1, Figure 2).

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

8 **The Performance of CircPanel, AFP and Their Combination for the Diagnosis of Small-HCC**

9 We then analyzed the performance of CircPanel, AFP and their combination (CircPanel+AFP) in the
10 diagnosis of Small-HCC (solitary, diameter ≤ 3 cm) and found that both the CircPanel and CircPanel+AFP
11 showed a higher accuracy than AFP in distinguishing individuals with Small-HCC from Non-HCC in all
12 three sets (CircPanel versus AFP: 0.862 [0.796–0.928] vs 0.680 [0.589–0.770], P=0.001 in the training set;
13 0.838 [0.776–0.900] vs 0.699 [0.613–0.785], P=0.011 in validation set 1 and 0.851 [0.799–0.903] vs 0.738
14 [0.671–0.805], P=0.009 in validation set 2. CircPanel+AFP versus AFP: 0.873 [0.817–0.929] vs 0.680
15 [0.589–0.770], P=0.001 in the training set; 0.874 [0.823–0.925] vs 0.699 [0.613–0.785], P=0.001 in
16 validation set 1 and 0.864 [0.818–0.910] vs 0.738 [0.671–0.805], P=0.002 in validation set 2). In addition,
17 the CircPanel and CircPanel+AFP did not show a significant difference in distinguishing Small-HCC from
18 Non-HCC (Table 2, Figure 3).

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

23 **The Performance of CircPanel for the Diagnosis of AFP-negative HCC and AFP-negative Small-HCC**

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

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Discussion

It is reported that plasma circRNA hsa_circ_0001445 is a fairly accurate marker for distinguishing HCC cases from healthy controls as well as liver cirrhosis or CHB patients.¹⁹ However, this was a single-center study with limited participants (104 HCC patients, 57 cirrhosis patients, 44 CHB patients, and 52 healthy controls).¹⁹

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 1155 participants. Importantly, the three circRNAs in the CircPanel proved to be secreted by HCC cells, and their expression in plasma was 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 of these findings make the CircPanel a compelling diagnostic biomarker.

There are a few limitations in the present study. First, all of 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 hepatectomy. The reason for this discrepancy is not clear at the present time and needs further exploration. 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, since the

1 expression of hsa_circ_0000976 and hsa_circ_0007750 in the HCC tissues was higher than in the ANL
2 tissues, their role in HCC progression awaits further investigation.

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

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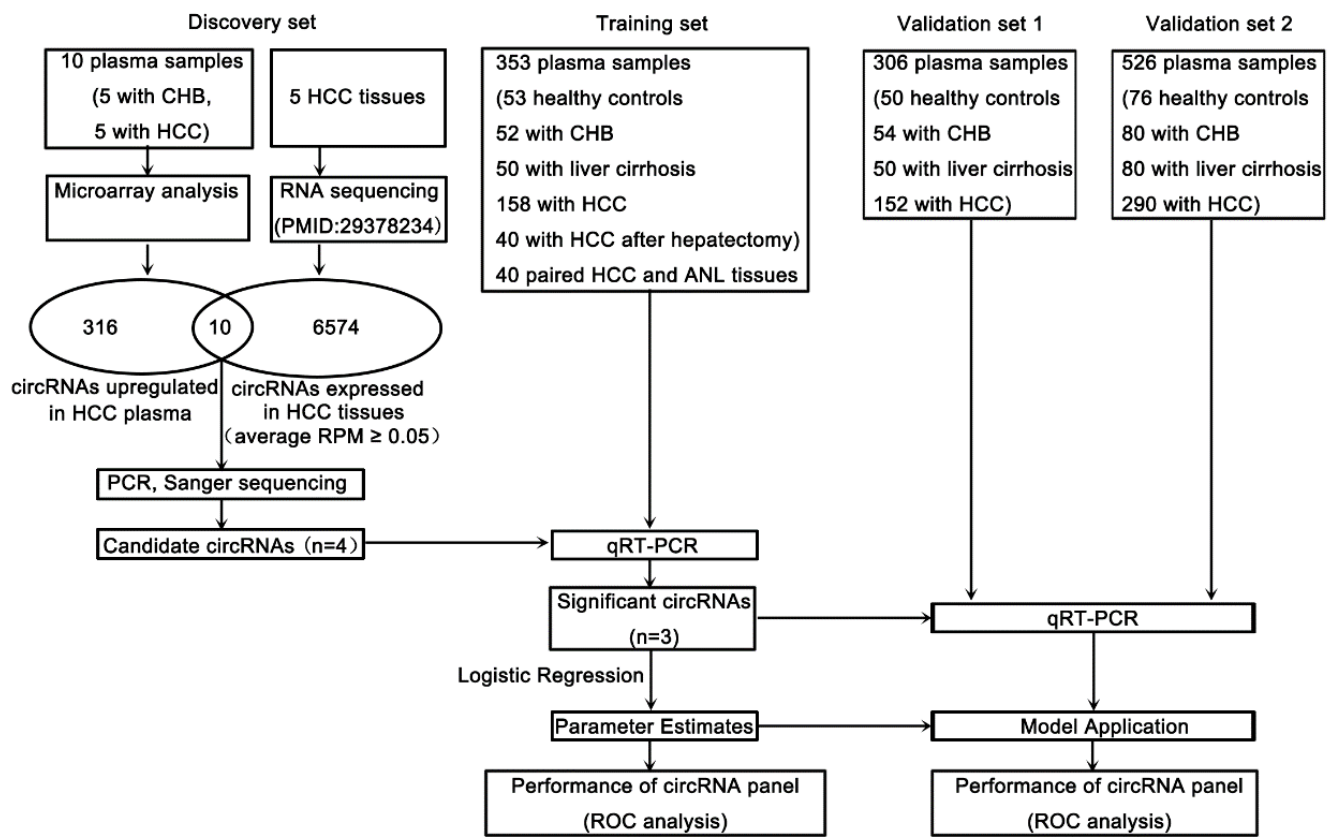
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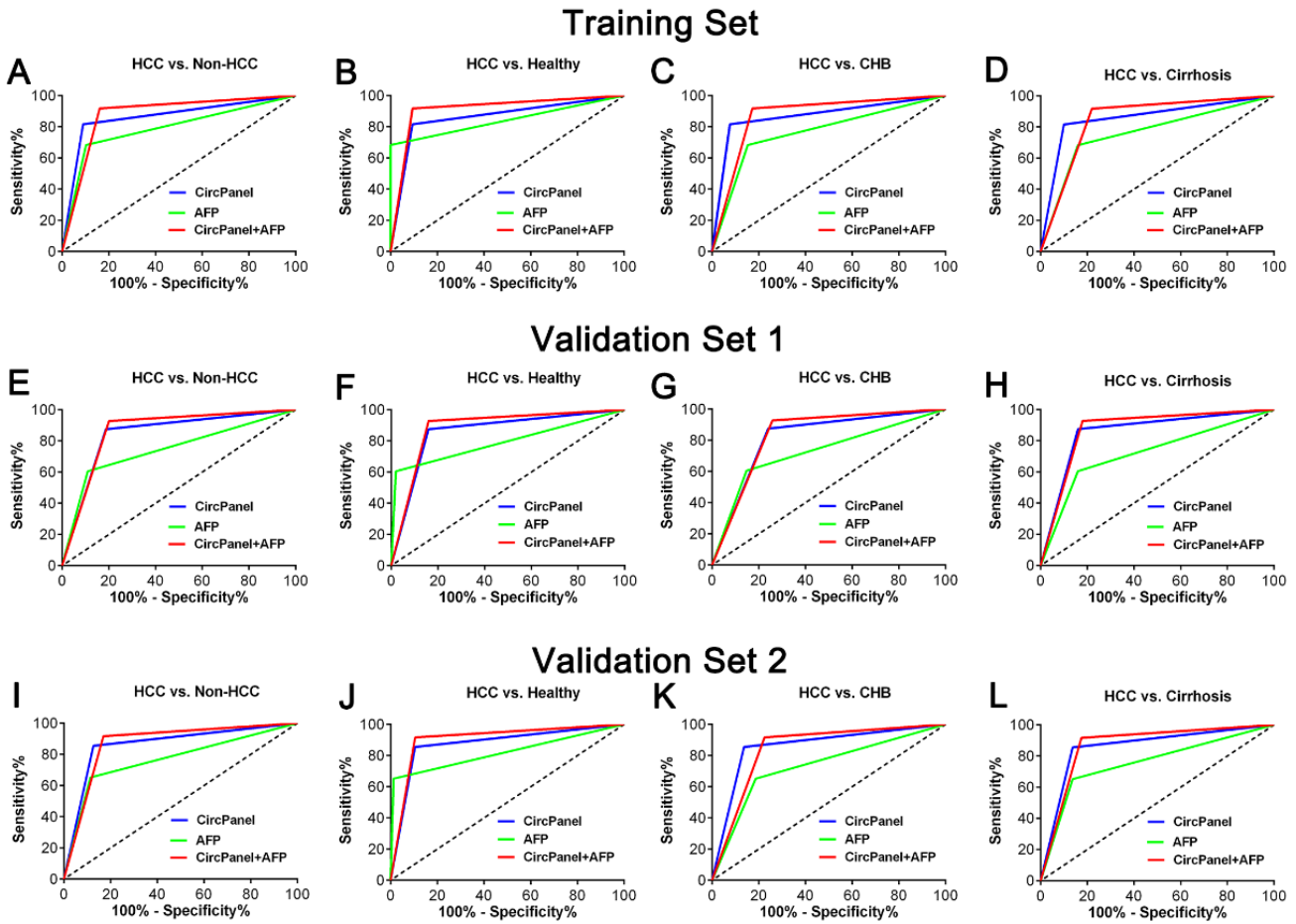
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5 **Figure 1. Study design.** Abbreviations: ANL, adjacent noncancerous liver; CHB, chronic hepatitis B; HCC,
6 hepatocellular carcinoma; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse-transcriptase
7 polymerase chain reaction; ROC, receiver operating characteristics.

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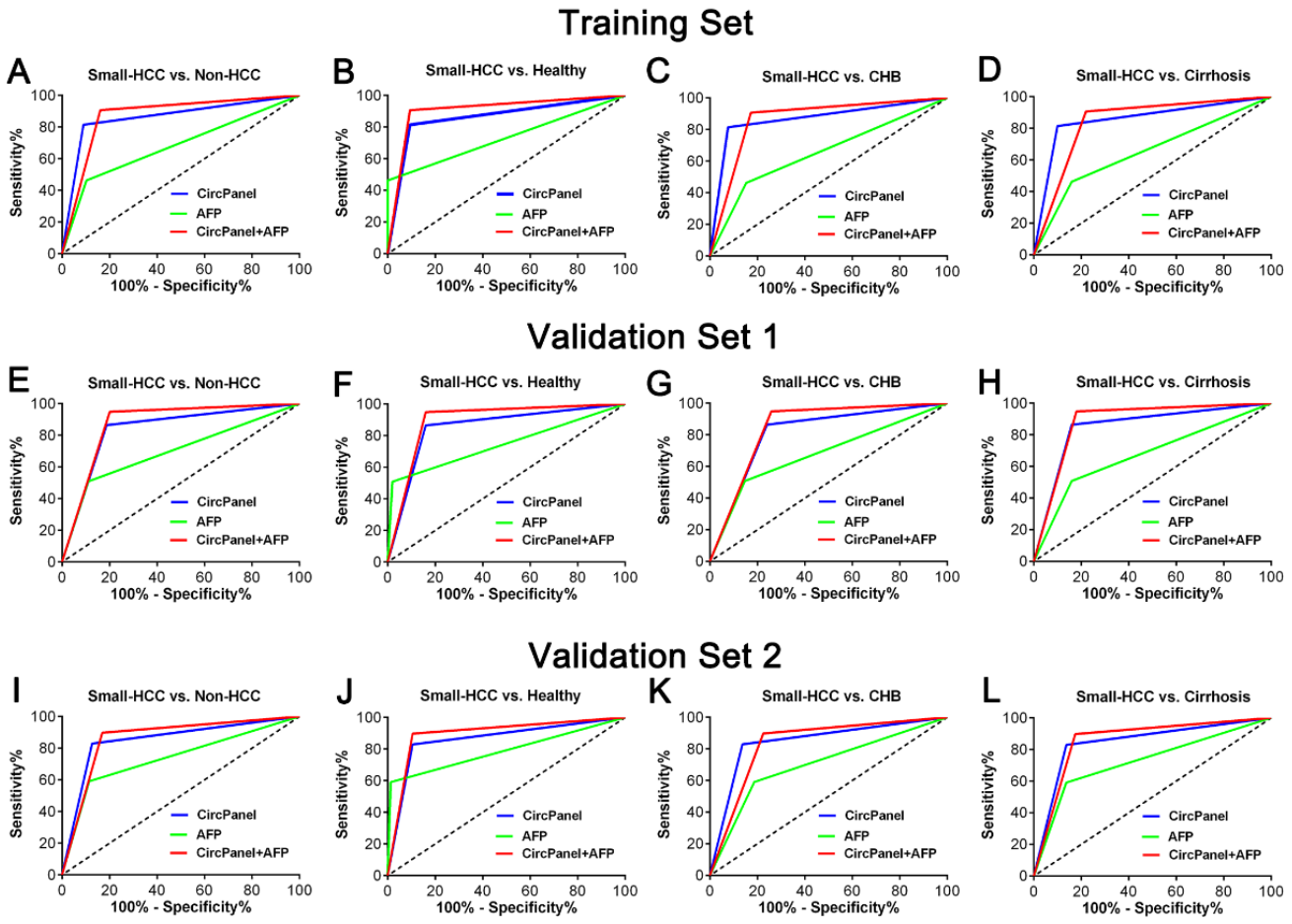
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4 **Figure 2. The performance of the CircPanel, AFP and their combination for the diagnosis of HCC in**
5 **the training set (A-D), validation set 1 (E-H) and validation set 2 (I-L).** The detailed diagnostic
6 performances are listed in Table 1. Abbreviations: AFP, alpha fetoprotein; CHB, chronic hepatitis B;
7 CircPanel, circRNA panel containing three circRNAs (hsa_circ_0000976, hsa_circ_0007750 and
8 hsa_circ_0139897); HCC, hepatocellular carcinoma.

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4 **Figure 3. The performance of the CircPanel, AFP and their combination for the diagnosis of**
5 **Small-HCC in the training set (A-D), validation set 1 (E-H) and validation set 2 (I-L). Thee detailed**
6 **diagnostic performances are listed in Table 1. Abbreviations: AFP, alpha fetoprotein; CHB, chronic hepatitis**
7 **B; CircPanel, circRNA panel containing three circRNAs (hsa_circ_0000976, hsa_circ_0007750 and**
8 **hsa_circ_0139897); HCC, hepatocellular carcinoma; Small-HCC, solitary, diameter ≤ 3 cm.**

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