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Plasma MicroRNA Panel to Diagnose Hepatitis B Virus–Related Hepatocellular Carcinoma

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
More than 60% of patients with hepatocellular carcinoma (HCC) do not receive curative therapy as a result of late clinical presentation and diagnosis. We aimed to identify plasma microRNAs for diagnosing hepatitis B virus (HBV) –related HCC.

Patients and Methods
Plasma microRNA expression was investigated with three independent cohorts including 934 participants (healthy, chronic hepatitis B, cirrhosis, and HBV-related HCC), recruited between August 2008 and June 2010. First, we used microarray to screen 723 microRNAs in 137 plasma samples for diagnosing HCC. Quantitative reverse-transcriptase polymerase chain reaction assay was then applied to evaluate the expression of selected microRNAs. A logistic regression model was constructed using a training cohort (n = 407) and then validated using an independent cohort (n = 390). Area under the receiver operating characteristic curve (AUC) was used to evaluate diagnostic accuracy.

Results
We identified a microRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) that provided a high diagnostic accuracy of HCC (AUC = 0.864 and 0.888 for training and validation data set, respectively). The satisfactory diagnostic performance of the microRNA panel persisted regardless of disease status (AUCs for Barcelona Clinic Liver Cancer stages 0, A, B, and C were 0.888, 0.888, 0.901, and 0.881, respectively). The microRNA panel can also differentiate HCC from healthy (AUC = 0.941), chronic hepatitis B (AUC = 0.842), and cirrhosis (AUC = 0.884), respectively.

Conclusion
We found a plasma microRNA panel that has considerable clinical value in diagnosing early-stage HCC. Thus, patients who would have otherwise missed the curative treatment window can benefit from optimal therapy.

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INTRODUCTION

Liver cancer is the second leading cause of cancer death in men and the sixth leading cause of cancer death in women worldwide. An estimated 748,300 new liver cancer cases and 695,900 liver cancer deaths occurred in 2008.[1] The poor prognosis of this disease is partially due to the lack of an effective means of early diagnosis. As a result, only 30% to 40% of patients with hepatocellular carcinoma (HCC) are candidates for potentially curative treatments at the time of diagnosis.[2] Discovery of an effective and reliable tool for early diagnosis of HCC would play a pivotal role in improving the prognosis of patients with HCC.

MicroRNA was initially identified in 1993 and has drawn significant attention in cancer research after it was linked to oncogenesis and tumor metastasis.[3-6] Many studies have demonstrated that microRNA expression profiles in HCC and nontumor tissue are significantly different.[7-13] Additional studies showed the existence of a large amount of stable microRNAs in human serum/plasma, which laid the foundation for studying the role of serum/plasma microRNAs in the diagnosis and prognosis of HCC.[14,15] In fact, differential expression of several serum microRNAs, including miR-16, miR-122, miR-21, miR-223, miR-25, miR-375, and let-7f in patients with HCC, patients with hepatitis B, and healthy individuals were reported recently.[9,16,17]

However, those studies were limited by one or more of the following factors: limited number of screened microRNAs, small sample size, failure to differentiate HCC from hepatitis B, and lack of independent validation.

Our study investigated plasma microRNA expression profiles (723 microRNAs) with independent validation in a large cohort of 934 participants, with the intention to identify a panel of microRNAs for the diagnosis of hepatitis B virus (HBV) –related HCC. The cohort included healthy individuals and patients with chronic hepatitis B (CHB), cirrhosis and HBV-related HCC.

PATIENTS AND METHODS

Study Design and Patients

Most HBV-related HCC cases involve two disease processes: the HCC and the disturbed liver function either from hepatitis or cirrhosis. It has been reported that large numbers of microRNAs are released to peripheral blood when liver cell damage occurs.[18] Identifying differential circulating microRNAs by contrasting HCC patients with controls as a whole group, which comprises healthy individuals, as well as the patients with hepatitis B and/or cirrhosis, might run the risk of under-representing the aberrant microRNAs from the healthy or cirrhosis groups. This potential risk could be due to the overwhelming numbers of circulating microRNAs released by the damaged liver cells in patients with hepatitis B. Therefore, the strategy of identifying differential microRNAs for HCC in this study was to choose candidate microRNAs on the basis of pairwise comparison of HCC versus healthy, HCC versus CHB, and HCC versus cirrhosis, respectively.

The 934 blood samples that met the eligibility criteria (Data Supplement Table 1), were collected from Zhongshan Hospital and Shanghai Public Health Clinical Center between August 2008 and June 2010. Those samples were allocated to three phases in chronological order (Fig 1).

1. Discovery phase. One hundred thirty-seven samples, each with 723 microRNAs, were screened with a microarray platform (patient characteristics are presented in Data Supplement Table 2). A Mann-Whitney test was performed to discover differentially expressed microRNAs in the three pairwise comparisons: HCC versus healthy, CHB, and cirrhosis, respectively. From the differentially expressed microRNAs (Data Supplement Tables 3A-3C), seven detectable microRNAs with P value $< .01$ and fold expression change ≥ 2 were identified between the HCC and healthy groups, seven detectable microRNAs with P value $< 10^{-9}$ and fold expression change ≥ 2 were identified between the HCC and CHB groups, and two detectable microRNAs with P value $< 10^{-9}$ and fold expression change ≥ 2 were identified between the HCC and cirrhosis groups. There was one pair of duplicate microRNAs in the HCC versus healthy and HCC versus CHB comparisons (Data Supplement Table 3D). Finally, 15 candidate microRNAs discovered via microarrays were selected for further testing by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

2. Training phase. The 15 microRNAs discovered via microarray were first tested with qRT-PCR in an independent cohort of plasma samples from 102 participants (Data Supplement Table 4). Seven microRNAs that were differentially expressed between the HCC and control groups (healthy, CHB, and cirrhosis) were further tested in an additional 305 participants. These 407 participants were used as the training set to construct the diagnostic microRNA panel based on the logistic regression model for the differentiation between the HCC group and the control group.

3. Validation phase. The parameters of the logistic model from the training phase were applied to an independent cohort of 390 samples for validating the diagnostic performance of the selected microRNA panel.

In each study phase, blood samples were obtained from four categories of participants including healthy individuals and patients with CHB, cirrhosis, and HCC. The investigational protocol was approved by local institutional review boards, and informed consents were obtained from all study participants.

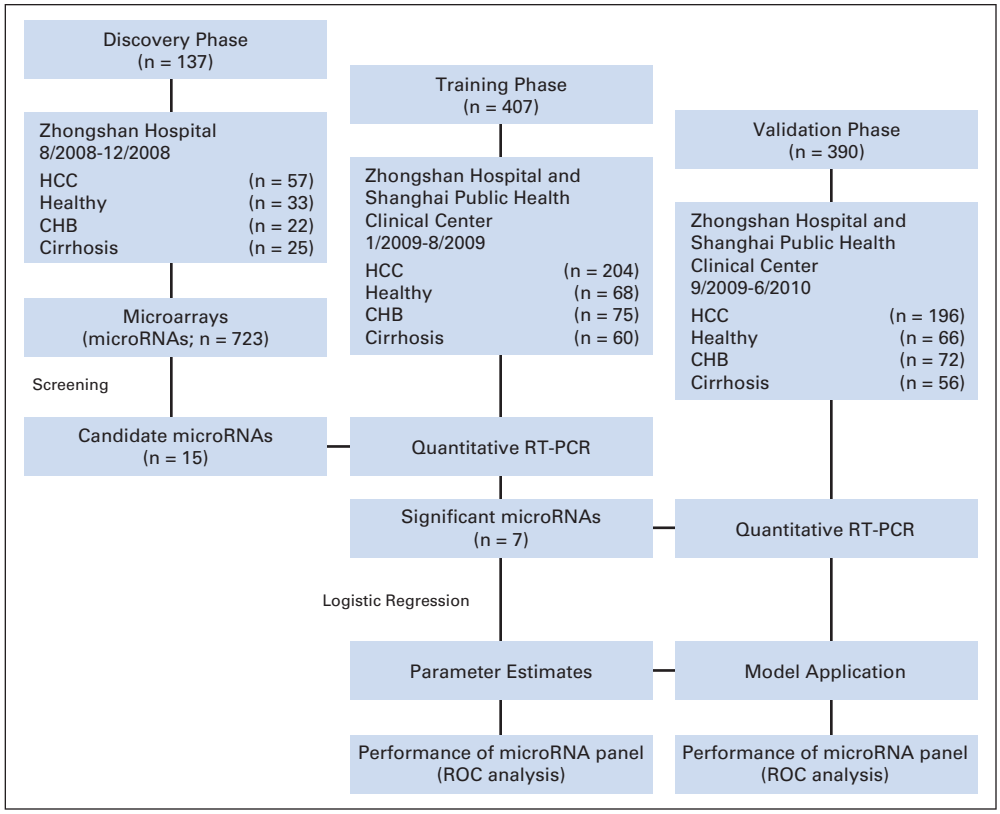

Fig 1. Study design. CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; ROC, receiver operating characteristics; RT-PCR, reverse transcriptase polymerase chain reaction.

Microarray and qRT-PCR

First, we used human microRNA microarrays 2.0 from Agilent Technologies (Santa Clara, CA) to identify candidate microRNAs for diagnosing HCC in 137 plasma samples (see Data Supplement for details of microarray hybridizations).

For testing of candidate microRNAs acquired on microarrays, qRT-PCR was performed using Taqman microRNA assays (Applied Biosystems, Foster City, CA). The assays were first performed on 102 samples for 15 candidates (miR-101, miR-122, miR-122*, miR-181d, miR-192, miR-194, miR-19a, miR-19b, miR-21, miR-223, miR-23b, miR-26a, miR-27a, miR-29c, and miR-801) that met the defined criteria (Data Supplement Fig 1). The expression level of miR-1228 was used as a stable endogenous control for normalization. All assays were carried out in triplicate. A microRNA that showed cycle threshold values above 35 in > 20% of the 102 samples were excluded from additional statistical analysis.

The assays were further applied on 695 samples for seven candidates (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) that were statistically differentially expressed in HCC compared with control (healthy, CHB and cirrhosis).

Statistical Analysis

For microarray analysis, the Mann-Whitney unpaired test was used for the three pairwise comparisons (HCC *v* healthy, HCC *v* CHB, and HCC *v* cirrhosis).[19] For the data obtained by qRT-PCR, the Mann-Whitney unpaired test was used for the comparison between HCC and control. A stepwise logistic regression model was used to select diagnostic microRNA markers based on the training dataset.[20] The predicted probability of being diagnosed with HCC was used as a surrogate marker to construct receiver operating characteristic (ROC) curve. Area under the ROC curve (AUC) was used as an accuracy index for evaluating the diagnostic performance of the selected microRNA panel.[21] MedCalc (version 10.4.7.0; MedCalc, Mariakerke, Belgium) software was used to perform the ROC and regression analysis. All *P* values were two sided.

RESULTS

Patient Characteristics

The characteristics of the study participants were presented in Table 1. There was no significant difference in the distribution of age and sex between the training and validation data sets for the four groups (healthy, CHB, cirrhosis, and HCC). For participants in the healthy and CHB groups, distribution of serum ALT was similar between the training and validation data sets. In the cirrhosis group, there were more patients with deranged liver function (ALT > 40) in the validation data set than in the training data set (61% *v* 35%, respectively; $P = .01$). In the HCC group, there were fewer patients with deranged liver function in the validation data set than in the training data set (24% *v* 34%, respectively; $P = .04$). Serum alpha feto-protein (AFP) level was well balanced between the training and validation data sets. In the HCC groups, all of the tumor characteristics except tumor size were similar between the two data sets. There were significantly fewer patients with tumors > 3 cm in the validation data set than in the training data set (54% *v* 67%, respectively; $P = .01$).

MicroRNA Screening and Testing

A microarray containing probes for 723 human microRNAs was initially used to screen the significant differential expression levels of microRNAs between the HCC and control groups (Data Supplement Table 3). Appendix Figure A1 (online only) illustrates the hierarchical clustering of the differentially expressed microRNAs in the pairwise comparison of the HCC and healthy groups, HCC and CHB groups, and HCC and cirrhosis groups, respectively. There were six microRNAs, including miR-122, miR-192, miR-194, miR-21, miR-23b, and miR-801, with significantly higher expression levels in the HCC group than in the healthy group (fold change = 2.0-6.7; $P < .01$; Data Supplement Table 3D). In contrast, miR-223 had a significantly lower expression level in the HCC group than in the healthy group (fold change = 0.5; $P < .01$). When compared with patients with CHB, patients with HCC had significantly lower expression levels of miR-101, miR-122*, miR-19a, miR-19b, miR-223, miR-27a, and miR-29c (fold change = 0.007-0.2; $P < 10^{-9}$). When compared with patients with cirrhosis, those with HCC had significantly lower expression levels of miR-181d and miR-26a (fold change = 0.1; $P < 10^{-9}$). In summary, 15 differentially expressed microRNAs were identified as candidates for further testing via qRT-PCR.

Differential Expression Profile of Seven Selected MicroRNAs

The 15 candidate microRNAs were first tested using an independent cohort of 102 plasma samples with qRT-PCR, and 12 of the 15 microRNAs passed the quality control. Seven of the 12 microRNAs had significantly different expression levels between the HCC and control groups (Data Supplement Table 4). The expression profile of those seven individual microRNAs was further evaluated with qRT-PCR on 305 additional plasma samples. The combined 407 plasma samples were used as the training data set for the construction of the microRNA panel for use in the diagnosis of HCC.

MicroRNA Expression Profile for HCC Versus Control in the Training Data Set

Low expression levels of miR-122, miR-223, miR-26a, and miR-27a were observed in patients with HCC compared with those in the control group (fold changes = 0.7, 0.3, 0.2, and 0.3 for miR-122, miR-223, miR-26a, and miR-27a, respectively; Table 2). The diagnostic accuracy of these four microRNAs, measured by AUC, was 0.553, 0.643, 0.665, and 0.638, respectively. High expression levels of miR-192, miR-21, and miR-801 were observed in patients with HCC compared with those in the control group (fold change = 1.4, 1.9, and 2.0 for miR-192, miR-21, and miR-801, respectively, Table 2). The corresponding AUCs were 0.569, 0.626, and 0.629, respectively. The multivariate *P* values for all of seven microRNAs were < .05 (Table 2).

Establishing the Predictive MicroRNA Panel

A stepwise logistic regression model to estimate the risk of being diagnosed with HCC was applied on the training data set (407 plasma samples). All of the seven microRNAs turned out to be significant predictors (Table 2). The predicted probability of being diagnosed with HCC from the logit model based on the seven microRNA panel, $\text{logit}(p = \text{HCC}) = -1.424 - 0.292 \times \text{miR-122} + 0.4511 \times \text{miR-192} + 0.6112 \times \text{miR-21} - 0.1796 \times \text{miR-223} - 0.2487 \times \text{miR-26a} - 0.3542 \times \text{miR-27a} + 0.209 \times \text{miR-801}$ was used to construct the ROC curve. The diagnostic performance for the established microRNA panel was evaluated by using ROC analysis. The AUC for the microRNA panel was 0.864 (95% CI, 0.826 to 0.895; sensitivity = 68.6%, specificity = 90.1%, Fig 2A).

Validating the MicroRNA Panel

The parameters estimated from the training data set were used to predict the probability of being diagnosed with HCC for the independent validation data set (390 plasma samples). Similarly, the predicted

Table 1. Characteristics of Study Participants in the Training and Validation Datasets

Variable	Training (n = 407) No.	%	Validation (n = 390) No.	%	*P*
Healthy count	68		66		
Age, years					.71
Mean	44		45		
SD	11		12		
Sex	35	49	43	65	.11
Male					
Female	33	51	23	35	
ALT					.76
≤ 40 U/L	56	82	53	80	
> 40 U/L	12	18	13	20	
AFP					
≤ 400 ng/mL	68	100	66	100	
CHB count	75		72		
Age, years					.93
Mean	39		39		
SD	13		14		
Sex					.06
Male	48	64	35	49	
Female	27	36	37	51	
ALT					.54
≤ 40 U/L	40	53	42	58	
> 40 U/L	35	47	30	42	
AFP					.55
≤ 400 ng/mL	75	100	68	94	
> 400 ng/mL	0	0	4	6	
Cirrhosis count	60		56		
Age, years					.13
Mean	53		50		
SD	13		10		
Sex					.98
Male	43	72	40	71	
Female	17	27	16	29	
ALT					**.01**
≤ 40 U/L	39	65	22	39	
> 40 U/L	21	35	34	61	
AFP					.11
≤ 400 ng/mL	60	100	53	95	
> 400 ng/mL	0	0	3	5	
HCC count	204		196		
Age, years					.95
Mean	53		53		
SD	12		12		
Sex					.53
Male	168	82	166	85	
Female	36	18	30	15	
ALT					**.04**
≤ 40 U/L	135	66	148	76	
> 40 U/L	69	34	48	24	
AFP					.31
≤ 400 ng/mL	135	66	139	71	
> 400 ng/mL	69	34	57	29	
Tumor size					**.01**
≤ 3 cm	68	33	90	46	
> 3 cm	136	67	106	54	
Multiple tumors					.11
Yes	34	17	45	23	
No	170	83	151	77	

(continued in next column)

Table 1. Characteristics of Study Participants in the Training and Validation Datasets (continued)

Variable	Training (n = 407) No.	%	Validation (n = 390) No.	%	*P*
BCLC stage					.25
0	26	13	36	18	
A	132	65	111	57	
B	31	15	38	19	
C	14	7	11	6	
D	1	0	0	0	
Edmonson grade					.78
I or II	138	68	135	69	
III	51	25	50	26	
No biopsy	15	7	11	6	
Vascular invasion					.61
Yes	71	35	70	36	
No	132	65	126	64	
No biopsy	1	0	0	0	

NOTE. Some percentages total > 100 because of rounding. Values in bold type are statistically significant.

Abbreviations: AFP, alpha fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; SD, standard deviation.

probability was used to construct the ROC curve. The AUC of the microRNA panel was 0.888 (95% CI, 0.852 to 0.917; sensitivity = 81.8%, specificity = 83.5%, Fig 2B).

The diagnostic performance of the microRNA panel in different Barcelona Clinic Liver Cancer (BCLC) stages was further evaluated (Figs 2C-2F). The corresponding AUCs for patients with BCLC stages 0, A, B, and C were 0.888, 0.888, 0.901, and 0.881, respectively. This indicated that the diagnostic performance of the microRNA panel was independent of disease status, which made it an optimal diagnostic tool.

The diagnostic accuracy of the microRNA panel was then evaluated according to AFP level. In the low AFP (< 400 ng/mL) group, the

Table 2. MicroRNA Profile and Diagnostic Performance in Training Dataset

MicroRNA Group	HCC Versus Control* Univariate *P*	Univariate Fold Change	Univariate AUC	Multivariate *P*
hsa-miR-122	.062	0.7	0.553	< .001
hsa-miR-192	.016	1.4	0.569	< .001
hsa-miR-21	< .001	1.9	0.626	< .001
hsa-miR-223	< .001	0.3	0.643	.0159
hsa-miR-26a	< .001	0.2	0.665	< .001
hsa-miR-27a	< .001	0.3	0.638	<.001
hsa-miR-801	< .001	2.0	0.629	.0026

NOTE. MicroRNA panel† AUC = 0.864 (95% CI, 0.826 to 0.895).

Abbreviations: AUC, area under the receiver operating characteristic curve; HCC, hepatocellular carcinoma.

*Control group includes healthy participants, patients with chronic hepatitis B, and patients with cirrhosis.

†logit(*P* = HCC) = −1.424 − 0.292 × miR-122 + 0.4511 × miR-192 + 0.6112 × miR-21 − 0.1796 × miR-223 − 0.2487 × miR-26a − 0.3542 × miR-27a + 0.209 × miR-801.

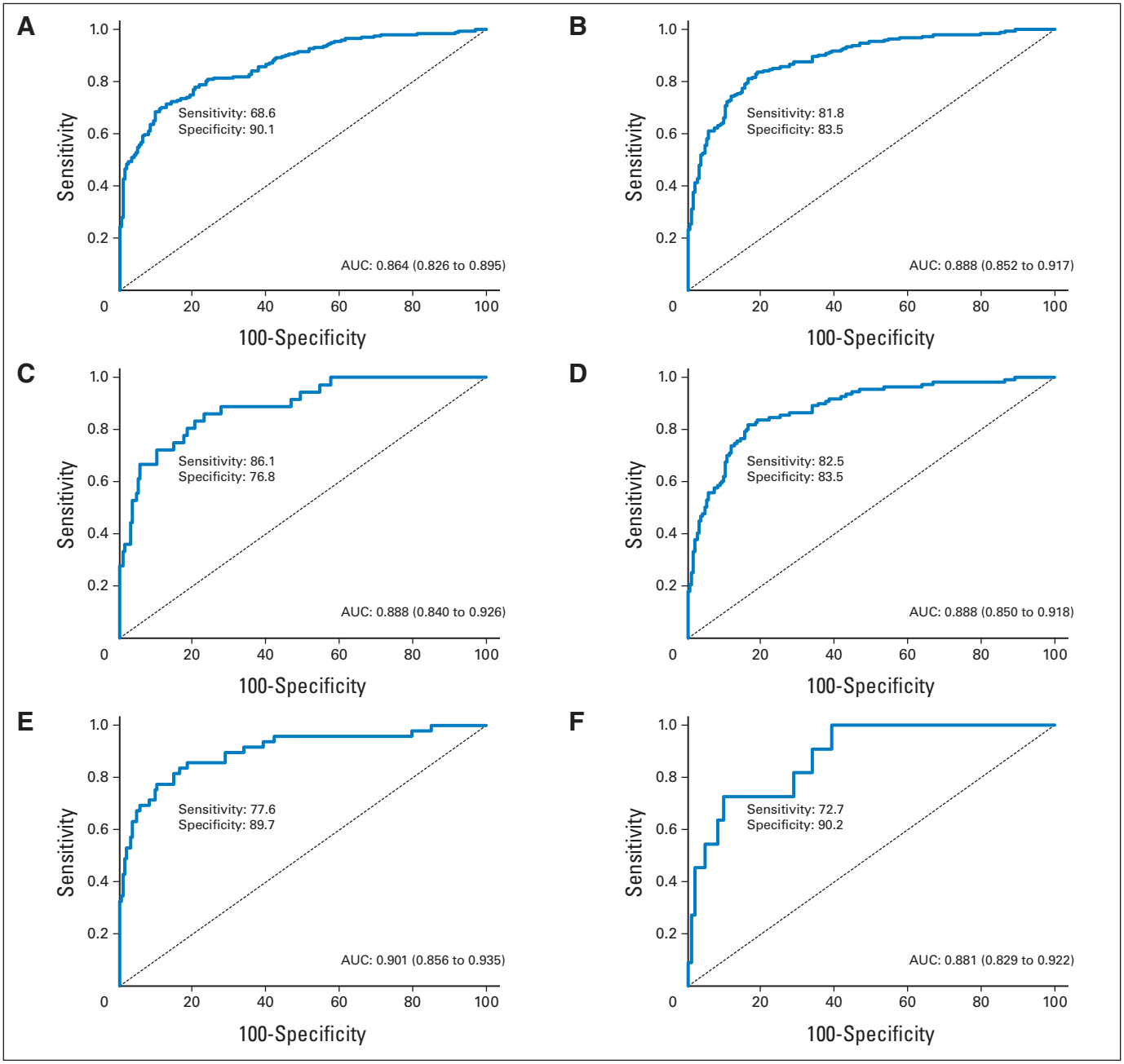

Fig 2. Receiver operating characteristic curve analysis for hepatocellular carcinoma diagnosis. Area under the curve (AUC) estimation for the microRNA panel in (A) the training set, (B) the validation set, (C) Barcelona Clinic Liver Cancer (BCLC) stage 0 and control, (D) BCLC stage A and control, (E) BCLC stage B and control, (F) BCLC stage C and control.

AUC of the microRNA panel was 0.879 (95% CI , 0.839 to 0.912; sensitivity = 77.7%, specificity = 84.5%; Data Supplement Fig 2A). In the elevated AFP (≥ 400 ng/mL) group, the AUC of the microRNA panel was 0.910 (95% CI, 0.867 to 0.942; sensitivity = 87.7%, specificity = 83.5%; Data Supplement Fig 2B).

The performance of the microRNA panel in differentiating the HCC group from the healthy, CHB, and cirrhosis groups was also evaluated, respectively (Fig 3). The analysis demonstrated that the microRNA panel had high accuracy in discriminating HCC from healthy (AUC = 0.941; 95% CI, 0.905 to 0.966; sensitivity = 83.2%, specificity = 93.9%), CHB (AUC = 0.842; 95% CI, 0.792 to 0.883; sensitivity = 79.1%, specificity = 76.4%) and cirrhosis (AUC = 0.884; 95% CI, 0.838 to 0.921; sensitivity = 75.0%, specificity = 91.1%).

DISCUSSION

Current methods for the diagnosis of HCC fall into two main categories: imaging and biomarker tests. However, the diagnostic performance of these modalities is unsatisfactory, particularly for the diagnosis of early-stage HCC. At this time, only 30% to 40% of patients with HCC are eligible for potentially curative intervention as a result of late clinical presentation and the lack of effective early

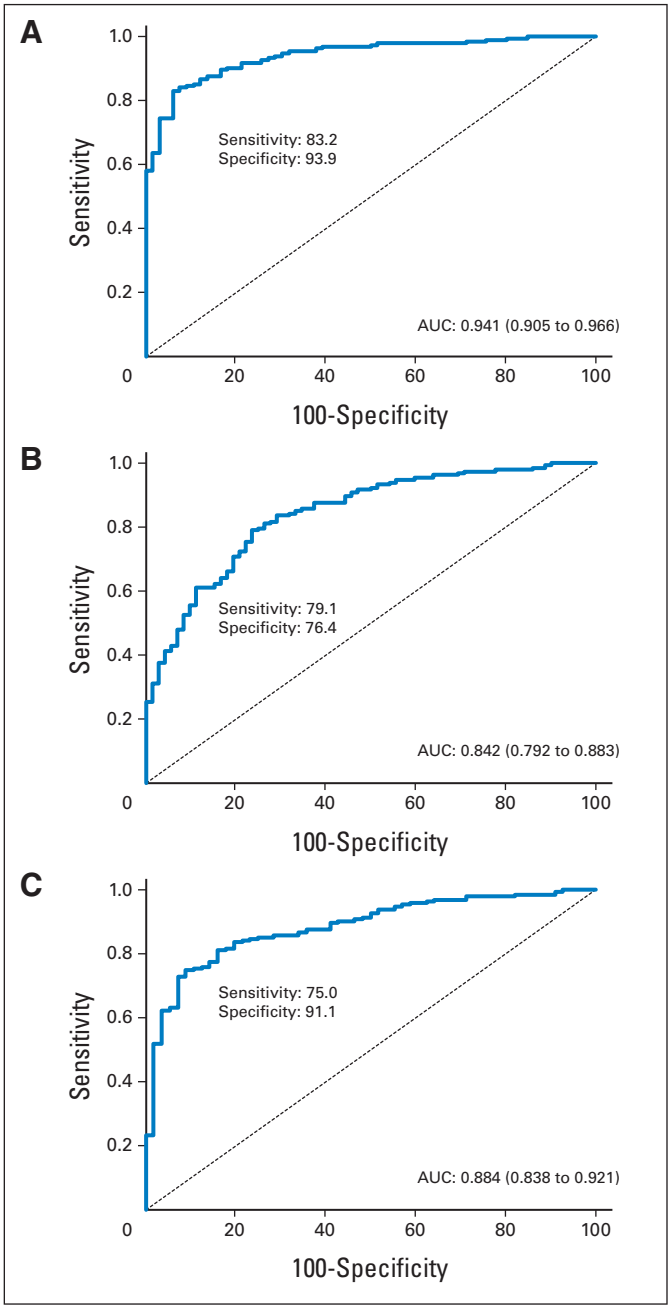

Fig 3. Receiver operating characteristic (ROC) curve analysis of the microRNA panel stratified by different groups in the validation set. ROC plots for the microRNA panel discriminating (A) the hepatocellular carcinoma (HCC) group from the healthy group, (B) the HCC group from the chronic hepatitis B group, (C) the HCC group from the cirrhosis group. AUC, area under the curve.

detection measures. AFP has been used for many years as a serum marker for HCC diagnosis and screening.[22,23] However, it has been recognized that AFP has poor sensitivity in the detection of HCC and that AFP levels often increase in the absence of HCC (chronic hepatitis or cirrhosis) as well.[24,25] Significant efforts to identify a better serum or plasma marker have met with limited success. The recent discovery of aberrant expression of microRNAs in HCC tissue paved the way for analyzing circulating microRNAs for the purpose of HCC diagnosis.

Our study revealed that plasma miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801 were potential circulating markers for diagnosing HCC. The microRNA panel with the seven microRNAs from the multivariate logistic regression model demonstrated high accuracy in the diagnosis of HCC, especially for patients with early BCLC stages (0 and A).

The association at the tissue level between HCC and four of the seven microRNAs (miR-122, miR-21, miR-223, and miR-26a) in our study has been previously reported. MicroRNA-122 is the most abundant microRNA in the liver, and the repression of miR-122 in HCC has been reported by many investigators.[7,26-29] Coulouarn et al[29] showed that the repression of miR-122 correlated with HBV-related HCC, tumor size, and differentiation grade. Furthermore, miR-122 repression was associated with poor prognosis and an increase in metastatic properties. Ladeiro et al[8] demonstrated a significant overexpression of miR-21 in HCC as compared with that in benign tumor or nontumor liver tissue. Overexpression of miR-21 has been shown to participate in down-regulating the expression level of phosphatase and tensin homolog.[10] Wong et al[13] demonstrated significantly repressed miR-223 in both HCC tissue and 18 HCC cell lines and identified Stathmin1 as a putative target of miR-223 in HCC. Our previous study showed that patients whose HCC tumors had lower miR-26a expression experienced worse survival but better response to interferon therapy.[30] Kota et al[31] found that the administration of miR-26a in a mouse model of HCC delivered by adeno-associated virus results in inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression. The differential expression profile of miR122, miR-21, and miR-223, and their potential role in carcinoma pathway had been reported by other studies. Further functional study is needed to confirm the role of miR-192 and miR-801. Although miR-27a has been found to be an oncogene in gastric cancer,[32] our study is the first to our knowledge to report the importance of the miR-27a expression profile, along with miR-192 and miR-801, in association with HCC.

At the circulating blood level, the diagnostic performance of miR-21, miR-122, and miR-223 in discriminating patients with HCC from a healthy group was reported by Xu et al[17]. However, their study failed to distinguish HCC from chronic hepatitis. Qu et al[16] found miR-16 to have moderate diagnostic accuracy of HCC, with sensitivity of 72.1% and specificity of 88.8%. In our study, miR-16 did show significant down-regulation in HCC as compared with CHB ($P < .01$), but up-regulation as compared with cirrhosis. However, it did not meet our candidate microRNA selection criteria at the microarray level. Li et al[9] reported the extraordinarily high diagnostic accuracy of the serum microRNA profiles for the diagnosis of HCC (AUC = 0.97-1.00) with microRNAs 10a, 125b, 223, 23a, 23b, 342-3p, 375, 423, 92a, and 99a. However, the need for different markers for different group comparisons with different critical values in their study (HCC *v* healthy, HCC *v* HBV, healthy *v* HBV, healthy *v* HCV, and HBV *v* HCV) raised concern about the robustness of the markers. Furthermore, these results have not been validated either internally or externally. Our study also confirmed that miR-23a, miR-23b, and miR-92a showed differential expression in the comparison of HCC versus CHB or HCC versus cirrhosis with the microarray platform.

However, these microRNAs were not further studied at qRT-PCR level, because they failed to pass our selection criteria.

Currently, there is no standard endogenous control for the circulating microRNA studies. The stable control (miR-1228) we used needs to be validated in more studies. Moreover, the patients' follow-up time in this study was only 1 to 3 years, limiting our current ability for prognostic analysis. In addition, our microRNA panel has yet to be validated across heterogeneous HCC patients, such as HCV-related HCC.

Compared with those studies of circulating microRNAs in diagnosing HCC,[9,16,17] our study is unique for the following reasons: First, we screened a large number of plasma microRNAs via microarrays, which enabled us to have better chance to identify potential diagnostic markers. Furthermore, we included not only HCC, healthy, and CHB groups but a cirrhosis group as well. It is well known that the pathogenesis of HCC is heterogenous and that multiple mechanisms of tumorigenesis could be involved (tumor suppressor gene, oncogene, viral effects, angiogenesis, etc). Nonetheless, we hypothesized that, similar to the adenoma-carcinoma sequence in colorectal cancer, the clinical pathway of most HBV-related HCC may follow the four states: healthy, hepatitis, cirrhosis, and HCC. Because of the long incubation time, microRNA disturbance might happen during any of the states (hepatitis, cirrhosis, or HCC) before the clinical/pathophysiological manifestation of HCC. Thus, all the representative differential microRNAs from HCC versus healthy, HCC versus hepatitis, and HCC versus cirrhosis should be considered. Failure to do so might be the source of the unsatisfactory discrimination of HCC from hepatitis or cirrhosis in other studies.[9,16,17] In addition, the microRNA panel identified in our study was validated by using a large, independent cohort from two medical centers.

In summary, we found a plasma microRNA panel in a large number of participants that differentiates HCC from healthy, CHB, and cirrhosis with a high degree of accuracy. Our study demonstrates that this plasma microRNA panel has considerable clinical value for the early diagnosis of HCC, so that more patients, who would have otherwise missed the curative treatment window, can benefit from the optimal therapy.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Appendix

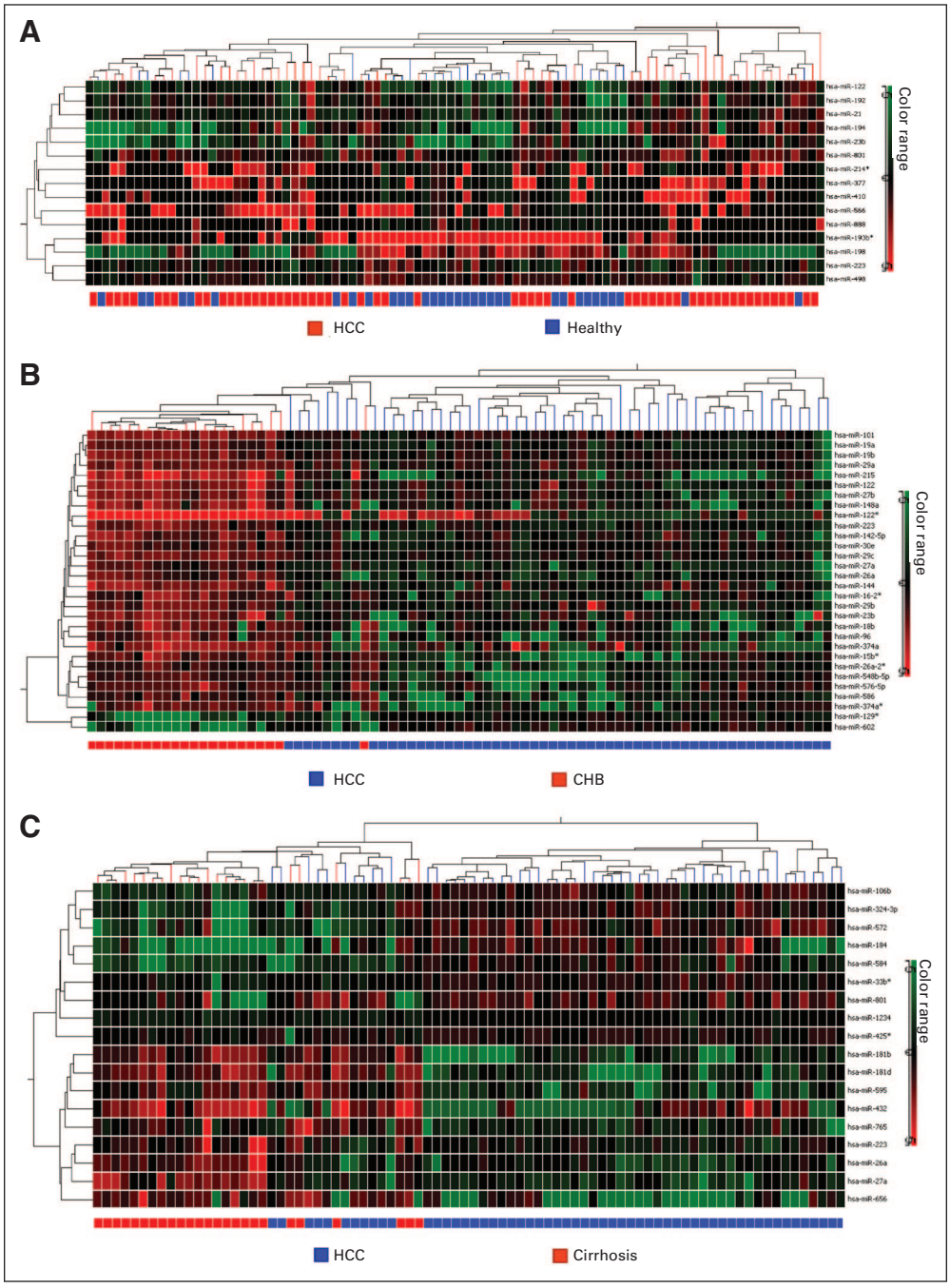

Fig A1. Hierarchical clustering of microRNA expression profiles in three group comparisons. (A) Hierarchical clustering of 15 microRNA expression profiles in the hepatocellular carcinoma (HCC) group versus the healthy group; (B) hierarchical clustering of 30 microRNA expression profiles in the HCC group versus the chronic hepatitis B (CHB) group; (C) hierarchical clustering of 18 microRNA expression profiles in the HCC group versus the cirrhosis group. The mean signal from biologic replicate samples was used for the clustering. Colored bars indicate the range of normalized log2-based signals.

Glossary Terms

Biomarker: A functional biochemical or molecular indicator of a biologic or disease process that has predictive, diagnostic, and/or prognostic utility.

Clustering: Organization of data consisting of many variables (multivariate data) into classes with similar patterns. Hierarchical clustering creates a dendrogram based on pairwise similarities in gene expression within a set of samples. Samples within a cluster are more similar to one another than to samples outside the cluster. The vertical length of branches in the tree represents the ex tent of similarity between the samples. Thus, shorter the branch length, the fewer the differences between the samples.

HCC (hepatocellular carcinoma): HCC is a type of adenocarcinoma. This is the most common form of liver cancer.

Logistic regression model: A multivariable prediction model in which the log of the odds of a time-fixed outcome event is related to a linear equation.

Microarray: A miniature array of regularly spaced DNA or oligonucleotide sequences printed on a solid support at high density that is used in a hybridization assay. The sequences may be cDNAs or oligonucleotide sequences that are synthesized in situ to make a DNA chip.

Quantitative RT-PCR: Quantitative RT-PCR consists of detecting PCR products as they accumulate. It can be applied to gene expression quantification by reverse transcription of RNA into cDNA, thus receiving the name of quantitative reverse transcriptase polymerase chain reaction. In spite of its name, quantitative, results are usually normalized to an endogenous reference. Current devices allow the simultaneous assessment of many RNA sequences.

ROC (receiver operating characteristic) curves: ROC curves plot the true positive rate (sensitivity) against the false-positive rate (1-specificity) for different cut-off levels of a test. The area under the curve is a measure of the accuracy of the test. An area of 1.0 represents a perfect test (all true positives), whereas an area of 0.5 represents a worthless test.

