

## Serum microRNA Profiles Serve as Novel Biomarkers for HBV Infection and Diagnosis of HBV-Positive Hepatocarcinoma

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### Abstract

Diagnosis of hepatitis B virus (HBV)-positive hepatocellular carcinoma (HCC), particularly HCC independent of cirrhosis etiology, presents a great challenge because of a lack of biomarkers. Here we test the hypothesis that expression profiles of microRNAs (miRNAs) in serum can serve as biomarkers for diagnosis of HBV infection and HBV-positive HCC. We recruited 513 subjects (210 controls and 135 HBV-, 48 hepatitis C virus (HCV)-, and 120 HCC-affected individuals) and employed a strategy of initial screening by Solexa sequencing followed by validation with TaqMan probe-based quantitative reverse transcription-PCR assay. First, because of a close link between chronic hepatitis B and HCC, we compared miRNA expression profiles in HBV serum with that in control serum and successfully obtained 13 miRNAs that were differentially expressed in HBV serum. This 13-miRNA-based biomarker accurately discriminated not only HBV cases from controls and HCV cases, but also HBV-positive HCC cases from control and HBV cases. Second, we directly compared miRNA expressions in HCC serum with those in controls and identified 6 miRNAs that were significantly upregulated in HCC samples. Interestingly, 2 of these miRNAs, miR-375 and miR-92a, were also identified by our first approach as HBV specific. When we employed 3 of these miRNAs (miR-25, miR-375, and let-7f) as biomarkers, we could clearly separate HCC cases from controls, and miR-375 alone had an ROC of 0.96 (specificity: 96%; sensitivity: 100%) in HCC prediction. In conclusion, our study demonstrates for the first time that serum miRNA profiles can serve as novel and noninvasive biomarkers for HBV infection and HBV-positive HCC diagnosis. *Cancer Res*; 70(23): 9798–807. ©2010 AACR.

### Introduction

Hepatitis B virus (HBV) infection is endemic in many Asian countries including China. There are 400 million people worldwide living with chronic HBV infection, of which more than 30% are Chinese (1). Chronic HBV infection continues to be a major contributor to morbidity and mortality despite the availability of vaccination programs in China and elsewhere (2). Although the implementation of hepatitis B surface antigen

(HBsAg) in the early 1970s greatly enhanced transfusion safety, transmission of blood components negative for HBsAg can still occur in the acute phase of infection during the seronegative window period or chronic stages of infection [i.e., "occult" HBV (OHB) infection] (3, 4). From a global perspective, chronic HBV infection is also the most important risk factor for hepatocellular carcinoma (HCC; ref. 5). Adults with chronic hepatitis B develop HCC at a rate of about 5% per decade, which is approximately 100-fold higher than the rate among uninfected populations (1). The geographic distribution patterns of HCC and HBV prevalence almost coincide, and developing countries contribute more than 80% of HCC, with China alone accounting for 55% (6). HCC is the third most common cause of cancer-related death, with 598,000 deaths per year (6). Such a high fatality rate shows the lack of specific noninvasive biomarkers and effective therapeutic strategies for this disease that is generally diagnosed at an advanced stage (7).

The development of HCC is a complex and multistep process. The molecular pathogenesis of HCC remains unclear, though the involvement of multiple genetic aberrations controlling hepatocyte proliferation, differentiation, and death has been suggested. Despite the presence of HBV DNA and hepatitis B e antigen in the blood, none could serve as indicator for the outcomes of HBV infection. The highly variable clearance rates and disease outcomes in persistently infected individuals cannot be fully explained by differences in

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viral load and monitored by clinical features. Thus, host genetic factors, the underlying molecular mechanisms and the corresponding surrogate biomarkers in relation to HBV infection need to be characterized to provide optimal management of HBV-related diseases from the time of initial diagnosis. Because cirrhosis from any cause often predisposes to HCC, it has been considered a premalignant condition. Indeed, the majority of patients worldwide with HCC have underlying some extent of cirrhosis (8) and HCC is a common cause of death among patients with compensated cirrhosis. However, some HCC cases may not have an etiology of cirrhosis. Recent epidemiologic data also show that in the United States and some European countries the mortality rates of cirrhosis clearly decreased whereas the mortality rates for HCC slowly increased (9, 10). Without a premalignant sign of cirrhosis, the cirrhosis-independent HCC is more difficult to diagnose.

Recently, alterations in expression of microRNAs (miRNAs) in both human and animal model have been linked to many forms of disease, especially cancer (11–22). miRNAs are a class of noncoding RNAs whose processed products are ~22 nucleotides in length and regulate gene expression (23). In light of the potential importance of miRNA in HCC, previous works defined the profiles of miRNA that are differentially expressed in a wide spectrum of HCCs and HCC cell lines (24–26), which opened a new avenue for the study of molecular mechanisms, diagnosis, and implementation of novel therapeutic targets of HCC. Very recently, we reported an exciting discovery that human serum/plasma contains a large amount of stable miRNAs and that the unique expression profile of serum miRNAs could serve as a fingerprint for various diseases (27). The potential of serum/plasma miRNAs as a novel noninvasive biomarker for disease diagnosis has been demonstrated by other studies (28–33).

In this study, we employed a strategy of Solexa initial screening followed by TaqMan probe-based quantitative reverse transcription-PCR (qRT-PCR) validation to analyze serum samples from 513 individual subjects, which were arranged in multiple training and testing sets. The results demonstrate that the unique expression pattern of serum miRNAs can serve as a sensitive, specific, and noninvasive biomarker for the diagnosis of HBV infection and HBV-positive HCC independent of cirrhosis etiology.

## Materials and Methods

### Patient characteristics and clinical features

This study was approved by the Institutional Review Board of Nanjing University and Nanjing Medical University, Nanjing, China, and the written informed consent was obtained from each participant.

As shown in Supplementary Table S1, for the identification of HBV-specific serum miRNAs, we recruited 160 controls [age, mean  $\pm$  SD: 37.52  $\pm$  12.09 years (range: 20–67); gender, 74 males and 86 females], 55 people with persistent asymptomatic HBV infection [age, mean  $\pm$  SD: 35.80  $\pm$  11.40 years (range: 21–67); gender, 33 males and 22 females], 80 chronic hepatitis B patients [age, mean  $\pm$  SD: 36.22  $\pm$  12.18 years (range: 20–70); gender, 55 males and 25 females], 65 HBV-positive/hepatitis C

virus (HCV)-negative HCC patients without etiology of cirrhosis [age, mean  $\pm$  SD: 53.43  $\pm$  9.10 years (range: 40–72); gender, 59 males and 6 females] and 48 chronic hepatitis C patients [age, mean  $\pm$  SD: 35.96  $\pm$  7.15 years (range: 24–62); gender, 39 males and 9 females] from the First Affiliated Hospital of Nanjing Medical University and the Nanjing Second Hospital (Nanjing, China) between September 2007 and July 2008. We also directly sequenced miRNAs in pooled serum samples from HCC and matched controls by Solexa and then validated the differentially expressed miRNAs using qRT-PCR. In this parallel experiment, 55 HBV-positive HCC patients without etiology of cirrhosis [age, mean  $\pm$  SD: 52.83  $\pm$  7.85 years (range: 42–70); gender, 46 males and 9 females] and 50 controls [age, mean  $\pm$  SD: 47.72  $\pm$  12.09 years (range: 36–72); gender, 42 males and 8 females] were recruited from the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). As shown in Supplementary Table S1, the exclusion criteria for all the groups included: (1) evidence of past or current infection by hepatitis A virus or hepatitis D virus; (2) birth or greater than 6 months residency in Qidong or Haimen Counties (Jiangsu Province, China); (3) systematic disease not related to HBV infection or pregnancy; (4) 1 or more parents or grandparents not of Han ethnicity; and (5) with more than 1 kind of hepatitis virus (HBV and HCV) infection. Furthermore, the HCC patients were all histopathologically diagnosed and all the blood samples were collected before any operation, chemotherapy, and/or radiation treatment.

Five milliliters of venous blood were collected from each participant at his/her first admission to the hospitals. To harvest cell-free serum, the blood was drawn into a sterile tube without anticoagulant. After leaving the tube in standing position for 20 minutes, samples were centrifuged at 20°C, 1,500g for 10 minutes, and the supernatant serum was quickly removed and stored immediately at  $-80^{\circ}\text{C}$  until analysis.

### RNA isolation and Solexa sequencing

RNA isolation and Solexa sequencing were described previously (27, 33). Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA). A synthetic, nonhuman miRNA, plant miR-168, was spiked into serum sample prior to RNA extraction. For Solexa sequencing, we used serum samples pooled from 30 controls or 30 cases (HBV or HCC). Small RNA molecules with the length less than 30 bases were ligated with a pair of Solexa adaptors and amplified for 17 cycles. The purified DNA was directly sequenced using Illumina's Solexa Sequencer. Finally, clean readouts were compared with the miRBase database (release 12.0) and the total copy number of the 2 sample groups was normalized against the spike-in plant miR-168 as an internal control.

### qRT-PCR assay

qRT-PCR assays to quantify the serum levels of miRNAs were conducted as previously described (27, 33). In each step from serum purification to qRT-PCR, equal volumes of serum were processed. The qRT-PCR assays were performed in triplicate using the same ABI 7300 machine. Expression levels of miRNAs were calculated using the  $C_T$  values. The ratio of the 2 groups of serum miRNAs were calculated by using the

equation  $2^{-\Delta\Delta G}$ , in which  $\Delta G = C_{T \text{ group1}} - C_{T \text{ group2}}$ . All primers used are available upon request.

### Statistical analysis

For average fold change of miRNAs in HBV patient serum compared with normal control serum, paired  $t$  tests were used, and values of  $P < 0.01$  were considered statistically significant. In addition to controlling variations throughout the steps, we standardized all the data to the mean of zero and the SD of 1 before cluster. We used hierarchical clustering in Cluster 3.0 with the complete linkage method. For risk scoring, the 5% or 95% (comparative to the reference group) reference interval of each miRNA, denoted as  $t$ , was set as the threshold to code the expression level of the corresponding miRNA for each sample. The risk score of each miRNA, denoted as  $s$ , was calculated as follows:

$$95\% \text{ threshold, } s_{ij} = \begin{cases} 0 & \text{if } r_{ij} < t_j \\ 1 & \text{otherwise} \end{cases} \quad (1)$$

$$5\% \text{ threshold, } s_{ij} = \begin{cases} 1 & \text{if } r_{ij} < t_j \\ 0 & \text{otherwise} \end{cases} \quad (2)$$

Here, we used  $i$  to denote the  $i$ th sample and  $j$  to denote the  $j$ th miRNA. Then, we presented frequency tables and receiver operating characteristic (ROC) curves to evaluate the diagnostic effects of the profiles. The statistical analyses were performed with Statistical Analysis System software (v.9.1.3; SAS Institute, Cary, NC).

## Results

Recent studies by us (27, 33) and others (30, 31, 34) demonstrate that human serum contains stable miRNAs, and the expression profile of these serum miRNAs can be used as potential fingerprints for various diseases. To obtain an expression profile of serum miRNAs that is specific for HBV infection or HBV-positive HCC, we employed a strategy including the initial screening by Solexa sequencing and the validation by qRT-PCR on an individual basis (Fig. 1). We started the search by comparing the miRNA expression profile in HBV serum with that in control serum. For Solexa sequencing, ~60 mL of serum pooled from 30 HBV patients or 30 healthy controls, was extracted for RNA followed by Solexa sequencing. As shown in Supplementary Table S2, there was notable alteration of miRNA expression in HBV compared with control serum. We considered miRNAs that satisfied 2 criteria as differentially expressed: (1) more than 50 copies in HBV serum by Solexa detection and (2) 20-fold higher expression in HBV compared with control serum. The analysis resulted in 21 differentially expressed miRNA in the serum of HBV cases compared with controls.

Because Solexa results were derived from the pooled serum samples, we next validated the expressions of these 21 miRNAs by TaqMan probe-based qRT-PCR assay using individual serum sample from the same 30 controls and 30 HBV cases. The expression value of each serum miRNA in HBV was converted to fold change as that of controls. For example, the expression level of miR-92a in HBV was  $13.6 \pm 3.5$ -fold of

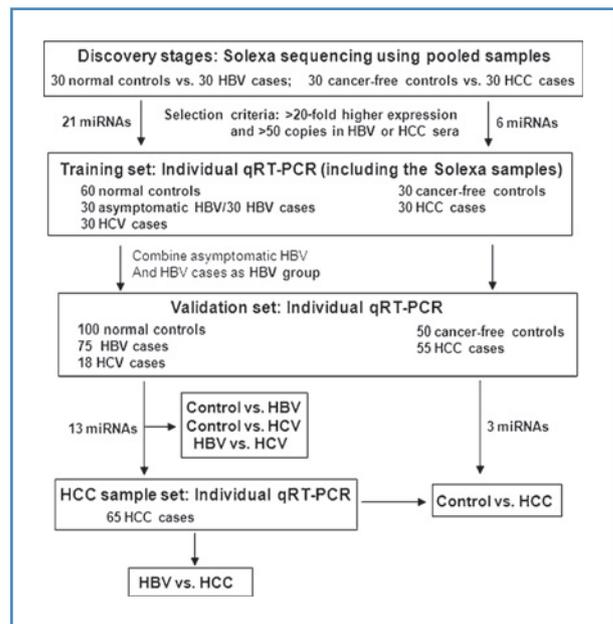


Figure 1. Overview of the design strategy.

its level in controls. By selecting miRNAs that have 3-fold higher expression in HBV compared with control serum, we identified total of 13 upregulated miRNAs, including miR-375, miR-92a, miR-10a, miR-223, miR-423, miR-23b/a, miR-342-3p, miR-99a, miR-122a, miR-125b, miR-150, and let-7c. As shown in Figure 2, the upregulation of these 13 miRNA expressions in

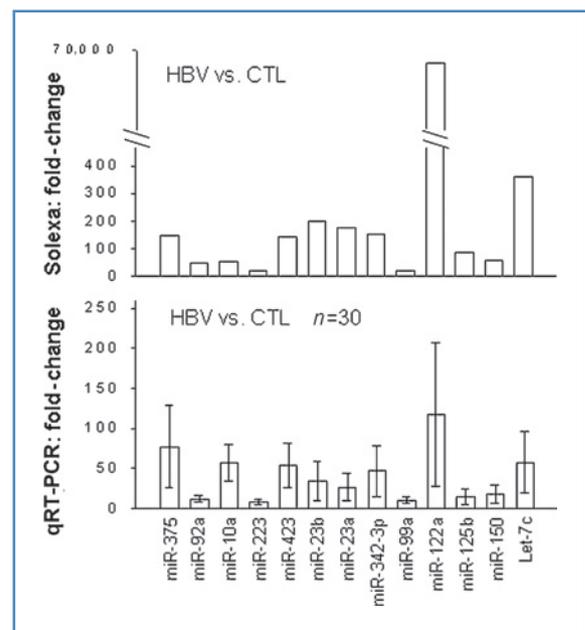
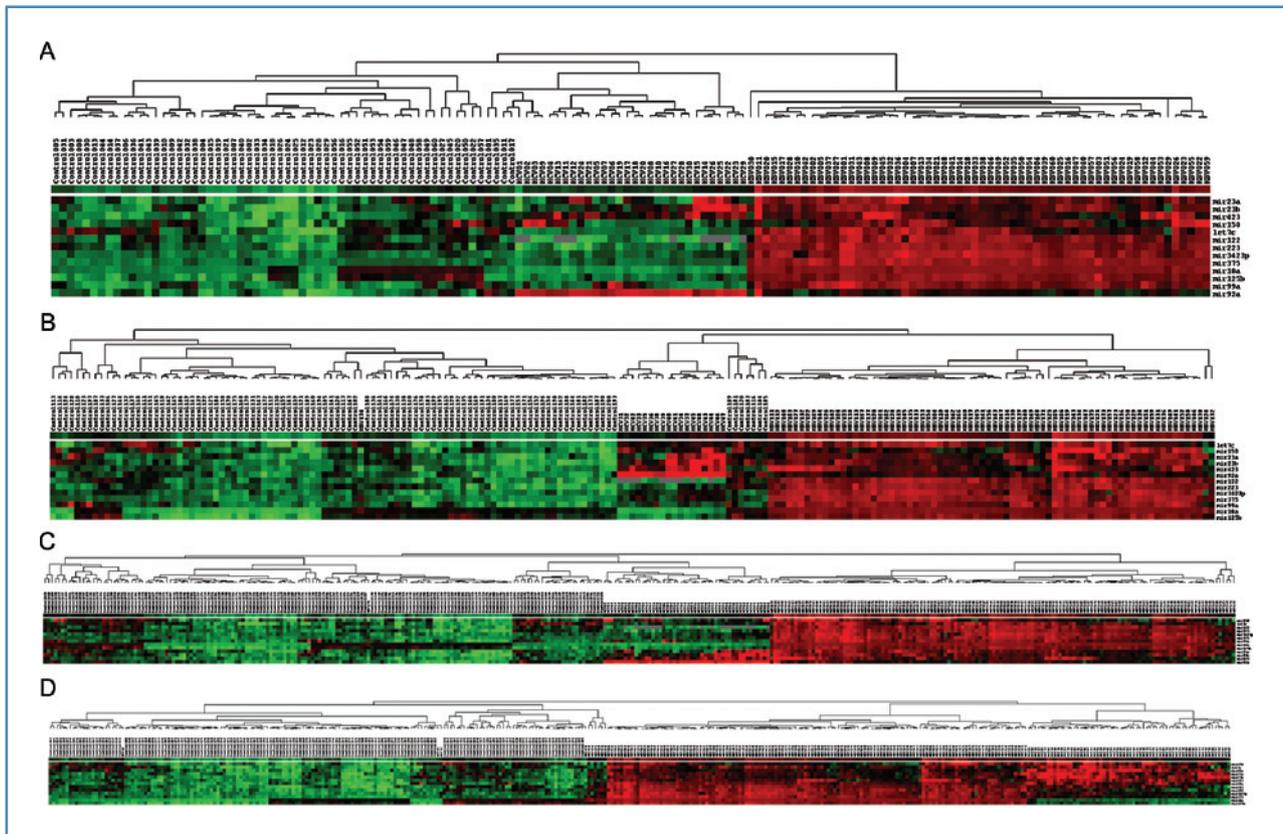


Figure 2. Upregulation of expression levels of 13 serum miRNAs in hepatitis B patients compared with healthy controls detected by Solexa sequencing and qRT-PCR assay. For Solexa sequencing, serum samples (~2 mL each) from 30 HBV patients or healthy controls were pooled together. For qRT-PCR assay, individual serum sample (~100  $\mu$ L each) was used. Data represented the mean  $\pm$  SD ( $n = 30$ ).



**Figure 3.** Separation of serum samples from control group, HBV group, HCV group, and HBV-positive HCC group by 13 serum miRNA-based biomarker. Clustering analysis based on 13 serum miRNAs was performed using samples from stage I (A), stage II (B), the combination of stage I and II (C), and stage III (D), respectively.

the serum of HBV cases, compared with those of controls, was largely consistent when detected by Solexa in pooled samples and qRT-PCR in individual samples.

We also examined the expressions of these 13 miRNAs in 30 persistent asymptomatic HBV-infection cases using qRT-PCR and observed no significant difference of miRNA expressions between chronic HBV and asymptomatic HBV carriers (Supplementary Table S3). Therefore, HBV patients and HBV carriers were combined into 1 HBV group and used for comparison hereafter (Fig. 1).

The expression profile of 13 serum miRNAs was further examined in a larger scale of samples. As shown in Supplementary Table S4, HBV, HCV, and HBV-positive HCC cases had a distinct expression profile of the 13 serum miRNAs when compared with normal controls. The discrimination of miRNA expression between the control and HBV groups was revealed by unsupervised clustering analysis in various stages. As shown in Figure 3A, the dendrogram generated by cluster analysis of 13-serum miRNA expression in 60 control, 60 HBV, and 30 HCV serum samples (training set) showed a clear separation of 3 groups on the basis of the 13-serum miRNA expression profile. To validate this result, another set of serum samples of 100 control, 75 HBV, and 18 HCV (validation set) were further detected for expressions of 13 miRNAs by qRT-PCR assay. The

samples were also clearly separated into 3 main classes (Fig. 3B). Together, the unsupervised clustering showed that these miRNA markers can separate the control, HBV and HCV groups with only 1 out of 135 HBV cases being classified incorrectly (Fig. 3C).

Because HBV infection is a major factor of HCC development, we further test whether these serum miRNAs can be markers for HBV-positive HCC diagnosis. For this experiment, we determined the expression profile of 13-miRNAs in serum of 65 HBV-positive, but HCV negative HCC patients and compared them with those from the control or other groups. All HCC patients showed no etiology of cirrhosis. As shown in Figure 3D, 13 serum miRNA-based biomarkers could separate HBV-positive HCC cases from HBV cases, HCV cases and control groups, with only 1 out of 135 HBV cases and 3 out of 65 HBV-positive HCC cases being misclassified.

To evaluate the diagnostic value for this 13-serum miRNA profile, we performed a risk scoring procedure on these data sets. By using the 5% or 95% reference interval of each miRNA expression value as risk scores, we constructed ROC curves and estimated the sensitivity and specificity for prediction. As shown in Table 1, miR-375 or miR-10a individually contribute to 97.5% area under curve (AUC) of ROC in separating the control and HBV groups, whereas the

**Table 1.** AUCs of single or multiple miRNAs to distinguish the samples between 4 groups

	Single C-P95 <sup>a</sup>	AUC	Multiple C-P95 <sup>a</sup>	AUC	Single C-P95 <sup>b</sup>	AUC	Multiple C-P95 <sup>b</sup>	AUC	Single HBV-P95 <sup>c</sup>	AUC	Multiple HBV-P95 <sup>c</sup>	AUC	Single HBV-P95 <sup>c</sup>	AUC
Stage I	miR-223	0.975	miR-223, miR-342-3p, miR-375/ miR10a	1.000	miR-92a	0.975	miR-92a, miR-423	0.995	miR-375	0.983	miR-375, miR-223/miR-10a/ miR-125b	0.992	miR-92a	0.975
Stage II	miR-375/ miR-10a	0.975	miR-375, miR-10a, miR-223/ miR-122	0.995	miR-92a/ miR-423	0.975	miR-92a, miR-423	1.000	miR-375/ miR-223/ miR-10a/ miR-125b	0.980	miR-223, miR-375/ miR-10a/ miR-125b	0.987	miR-92a	0.980
Merged stage I/II	miR-375/ miR-10a	0.975	miR-375, miR-10a, miR-223/ miR-122/ miR-342-3p, miR-423	1.000	miR-92a	0.975	miR-92a, miR-423	0.996	miR-375	0.982	miR-375, miR-223/ miR-10a/miR-125b, miR-342-3p, miR-99a	0.995	miR-92a	0.978
Stage III	miR-23b	0.967	miR-23b, miR-423, miR-375, miR-23a, miR-342-3p	0.999	miR-10a	0.978	miR-10a, miR-125b	0.992	miR-92a	0.839	miR-92a, miR-23b, miR-23a	0.928		

C-P95: More than the upper 95% reference interval of each miRNA in the group; HBV-P95: Less than the 5% reference interval of each miRNA in the HBV group; HBV-P95: More than the upper 95% reference interval of each miRNA in the HBV group.

<sup>a</sup>Control versus HBV.

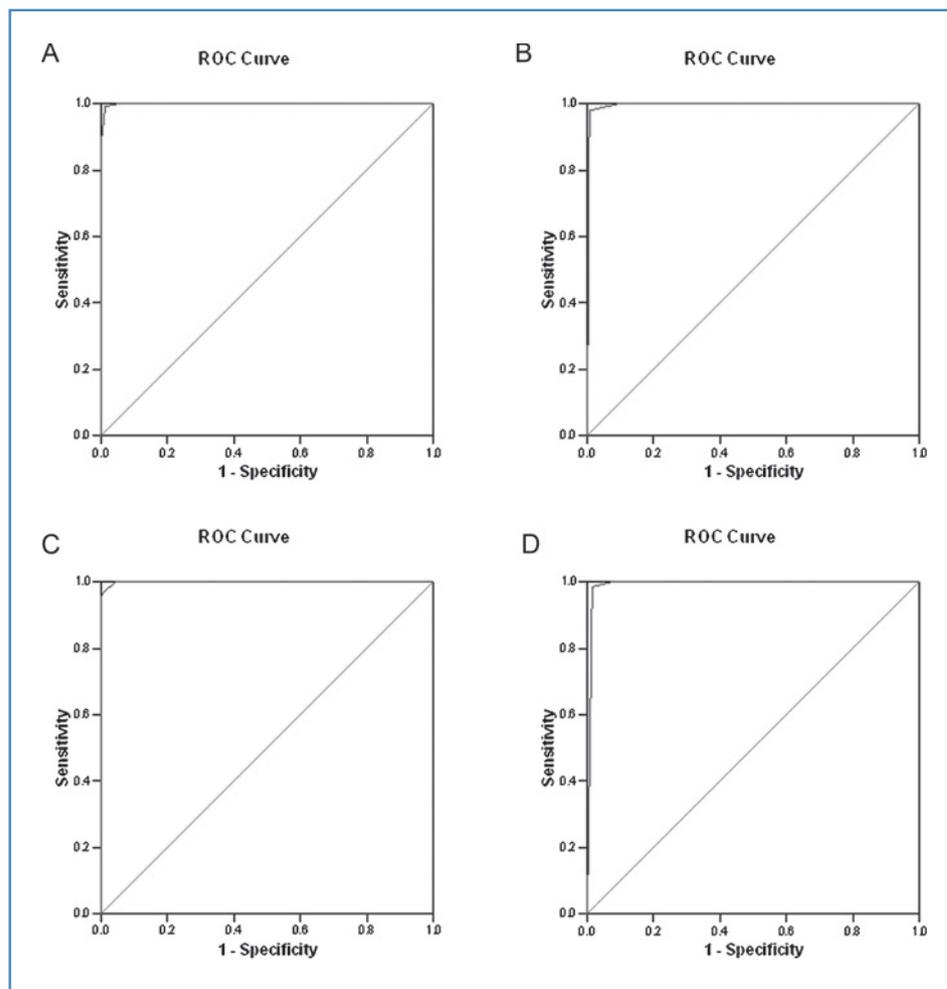
<sup>b</sup>Control versus HCV.

<sup>c</sup>HBV versus HCV.

<sup>d</sup>Control versus HBV-positive HCC.

<sup>e</sup>HBV versus HBV-positive HCC.

**Figure 4.** ROC curves between control and HBV groups (A), control and HCV groups (B), control and HBV-positive HCC groups (C), and HBV and HBV-positive HCC groups (D). A, control versus HBV (miR-375, miR-10a, miR-223, and miR-423; AUC:  $99.9 \pm 0.1\%$ ; sensitivity: 99.3%; specificity: 98.8%); B, control versus HCV (miR-92a and miR-423; AUC:  $99.6 \pm 0.4\%$ ; sensitivity: 97.9%; specificity: 99.4%); C, control versus HBV-positive HCC (miR-23b, miR-423, miR-375, miR-23a, and miR-342-3p; AUC:  $99.9 \pm 0.1\%$ ; sensitivity: 96.9%; specificity: 99.4%); D, HBV versus HBV-positive HCC (miR-10a and miR-125b; AUC:  $99.2 \pm 0.6\%$ ; sensitivity: 98.5%; specificity: 98.5%).

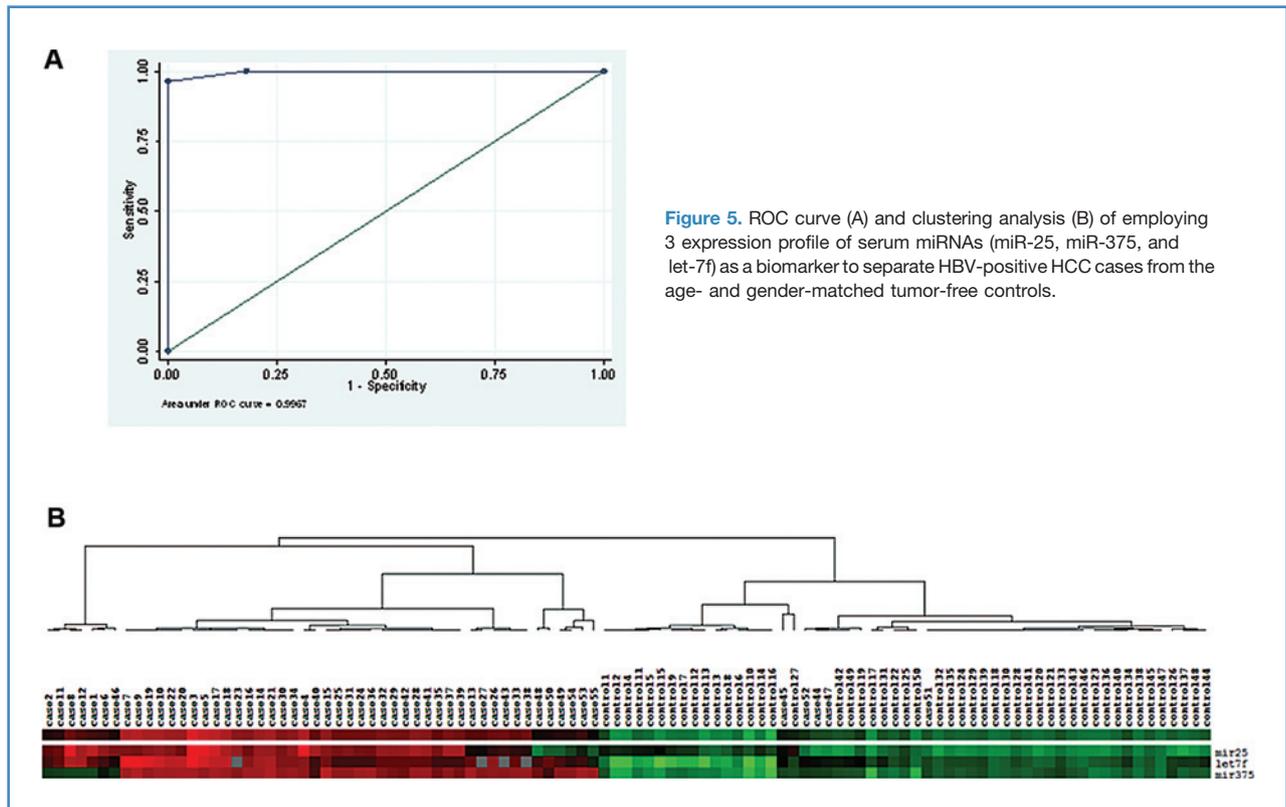


combination of miR-375, miR-10a, miR-223 (miR-122a or miR-342-3p), and miR-423 resulted in 100% AUC. Similarly, miR-92a individually contributes to 97.5% AUC and the combination of miR-92a and miR-423 resulted in 99.6% AUC in separating the control and HCV groups. In contrast, miR-375 and miR-92a contribute to distinguishing the HBV and HCV groups in an oppositely expressed direction. The combination of miR-23b, miR-423, miR-375, miR-23a, and miR-342-3p resulted in 99.9% AUC in separating the HBV-positive HCC group from the control group. Individually, miR-10a and miR-92a contributed to discrimination of the HBV group and HCC group in an oppositely expressed direction.

As shown in Figure 4A, when we use 4 markers (miR-375, miR-10a, miR-223, and miR-423) to separate the control and HBV groups, the AUC was  $99.9 \pm 0.1\%$  (sensitivity: 99.3%; specificity: 98.8%). Similarly, 2 markers (miR-92a and miR-423) could separate the control and HCV groups with a high specificity and sensitivity (AUC:  $99.6 \pm 0.4\%$ ; sensitivity: 97.9%; specificity: 99.4%; Fig. 4B). As shown in Figure 4C, the control and HBV-positive HCC group could be clearly separated by 5 markers (miR-23b, miR-423, miR-375, miR-

23a, and miR-342-3p; AUC:  $99.9 \pm 0.1\%$ ; sensitivity: 96.9%; specificity: 99.4%). Similarly, the HBV and the HBV-positive HCC group could be separated by 2 markers (miR-10a and miR-125b; AUC:  $99.2 \pm 0.6\%$ ; sensitivity: 98.5%; specificity: 98.5%; Fig. 4D).

The expression profile of serum miRNAs as biomarkers for HBV-positive HCC diagnosis was also tested by directly comparing miRNA profile in pooled serum samples from 30 HCC cases with that from 30 age- and gender-matched tumor-free controls via Solexa sequencing. After validation of the HCC differentially expressed miRNAs selected by Solexa screening using another serum samples consisting of 55 HCC and 50 controls, we identified 6 serum miRNAs (miR-1, miR-25, miR-92a, miR-206, miR-375, and let-7f) that were significantly altered in HCC cases compared with the controls (Supplementary Table S5). Interestingly, as can be seen in Figure 2 and supplementary Table S4, 2 of these 6 miRNAs, miR-375 and miR-92a, were also identified by directly comparing HBV with control serum. As shown in Table 1, both miR-375 and miR-92a were HBV-specific miRNAs and significantly contributed to distinguishing HBV-positive HCC cases from HBV, HCV, and control groups. Identification of the HBV-specific miRNAs



**Figure 5.** ROC curve (A) and clustering analysis (B) of employing 3 expression profile of serum miRNAs (miR-25, miR-375, and let-7f) as a biomarker to separate HBV-positive HCC cases from the age- and gender-matched tumor-free controls.

as an HCC biomarker confirms a tight linkage between HBV infection and HCC and suggests that certain HBV-specific miRNAs may serve as biomarkers for HBV-positive HCC. As expected, directly comparing HBV-positive HCC serum with control serum by Solexa sequencing also resulted in identification of other miRNA, such as miR-25 and let-7f, that were upregulated in HCC but not changed or even decreased in HBV serum. These miRNAs might be involved in some processes of HCC development that are independent to chronic HBV infection, therefore would provide additional biomarkers for HCC testing. As shown in Figure 5A, when 3 of these 6 serum miRNAs, miR-375, miR-25, and let-7f, were used as biomarkers, the HBV-positive HCC group could be clearly separated from the control group (AUC:  $99.67 \pm 0.15\%$ ; sensitivity: 97.9%; specificity: 99.1%). Serving as a biomarker, miR-375 alone had a ROC 0.96 (specificity: 96%; sensitivity: 100%) in HCC prediction. The unsupervised clustering further showed that using 3 serum miRNA-based biomarkers we can discern the controls and HCC cases with only 5 out of 55 HCC cases being classified incorrectly (Fig. 5B).

## Discussion

In the diagnosis of chronic liver diseases including HCC, major efforts have been made to develop noninvasive serum biomarkers. Although remarkable advances have been made,

the reliability of these biomarkers is still debatable. In the field of cancer diagnosis, the markers that are used in clinical practice are mostly organ specific. Up to now, alpha-fetoprotein (AFP) is the only available marker for HCC diagnosis. However, the specificity of AFP is low, especially in the context of chronic liver diseases (35). Therefore, novel biomarkers for early HCC diagnosis are greatly needed.

In this study, we provide a "proof-of-principle" approach to identify a particular disease-specific serum miRNA profile. This approach includes a Solexa sequencing of pooled serum samples followed by multiple qRT-PCR validation sets at the individual level. Employing this approach, we identified a unique expression profile for HBV-related serum miRNAs and HCC-related serum miRNAs, respectively. Given the fact that chronic HBV-infection is closely linked to HBV-positive HCC, several HBV-specific miRNAs, such as miR-375 and miR-92a, were also differentially expressed in HCC serum compared with the control. In agreement with this, the 13 HBV-specific serum miRNA-based biomarker can clearly separate not only HBV cases from controls and HCV cases but also HBV-positive HCC from controls, HBV cases, and HCV cases (Fig. 3). By directly comparing HCC serum with the matched controls, we obtained 6 serum miRNAs that were significantly altered in HCC cases. This panel of miRNAs includes HBV-specific miRNA, such as

miR-375 and miR-92a, and miRNAs that are not significantly altered by chronic HBV infection such as miR-25 and let-7f. These results suggest that both HBV infection and other HBV-independent factors are likely involved in HCC development.

As a critical stage of HCC development, cirrhosis often has been considered a premalignant sign of HCC. Because all the HCC cases recruited in this study had no etiology of cirrhosis, it remains unknown whether our serum miRNA-based biomarkers derived from this study can distinguish HCC from cirrhosis. However, our initial screening of cirrhosis and HCC by Solexa sequencing in an independent study has shown that cirrhosis-specific serum miRNA expression profile is significantly different with that of HCC cases or controls (Supplementary Table S6), strongly arguing that cirrhosis and HCC are 2 quite different diseases and implicating that a unique miRNA profile can be identified as biomarker for cirrhosis. Although the underlying molecular mechanisms need to be carefully evaluated in future studies, our approach provides promising noninvasive biomarkers in surveillance of HBV infection and HBV-positive HCC. Giving the success of Solexa sequencing and qRT-PCR in identifying serum miRNAs specific for HBV and HBV-positive HCC, our approach will be greatly useful in future identification of serum miRNA-based biomarkers. For example, a similar approach can be employed to identify metastasis- and survival-specific serum miRNA profiles to facilitate the early diagnosis and outcome prediction of HCC.

Although the source of serum miRNAs and the mechanisms that control the biogenesis of serum miRNAs remain unknown, we speculate that miRNAs may enter the circulation via active secretion from blood cells or tissues/cells that are affected by diseases. Therefore, a comparison of miRNA expression pattern between serum and tissue/cell would provide additional evidence supporting serum miRNAs as reliable diagnostic biomarkers. The differential expression profile of miRNAs in HCC tissues has been recently reported (24, 36–40) and several differentially expressed miRNAs such as miR-21 (41), miR-221 (42), miR-199a (40), and miR-224 (39), showed a potential as HCC markers in tissue and cell samples. Datta et al. (43) reported that miR-1 and its role in hepatocellular carcinogenesis could be silenced by methylation. In addition, the studies by Varnholt et al. (25) and Braconi et al. (22) showed unique expression pattern of tissue miRNAs related to HCV-associated HCC. With the identification of the potential target genes of these HCC-associated miRNAs, it may shed light into our understanding of tumorigenesis and development of HCC. For example, serum miR-375 contributed to separation of both HBV-infection cases and HBV-positive HCC cases from the controls. In tissue samples, the low expression of miR-375 was also observed in benign hepatocellular adenomas and HCC mutated for  $\beta$ -catenin (30). Our data showed that serum miR-92a could help to distinguish HBV infection from HCV infection and HBV-positive HCC from HBV infection (Table 1), respectively. In tissue study, miR-92a was reported

to be increased in HBV-positive HCC (24) suggesting that the miR-17–92 polycistron may represent another important etiologic agent in HCC initiation. In addition, miR-10a, miR-125b, miR-122a, and miR-223 were reported to decrease in HCC tissues and cell lines (24), which is consistent with our observations in the serum. Our study showed that miR-25, in which expression was not altered by HBV infection, could serve as a biomarker for HCC diagnosis (Fig. 5). By studying tissue miRNAs in Barrett's esophagus, Kan et al. (44) reported that miR-25 serves as an oncogene by targeting Bim. Also, the study by Kuhn et al. (45) showed a broad role miR-25 may play in modulating expression of inflammatory mediators such as RANTES, eotaxin, and TNF $\alpha$ .

Results from this study also show that although 1 particular miRNA in serum may help distinguish different sample sets (i.e., miR-375/miR-10a for control and HBV-infected subjects, and miR-92a for control and HCV-infected subjects), a combination of multiple miRNAs can offer more specific testing. The fact that persistent asymptomatic HBV infection persons share similar expression profiles with that of HBV patients suggests that the definition of "disease state" by serum miRNAs could be at a very early stage without pathophysiologic syndrome. In addition, because the detection of serum miRNAs is not directly dependent on body immune response, such as antigen or antibody generation, the serum miRNA profile may be also useful to detect OHB infection (3). The patients under OHB infection generally contain HBV DNA in blood or liver tissues but are negative for HBsAg (46). Because the current blood screening practices depend on HBsAg detection, OHB can be an overlooked source of HBV transmission.

An understanding of the molecular mechanisms by which miRNAs promote HCC development may aid early diagnosis and treatment of this highly malignant type of tumor. Many verified or predicted mRNAs targeted by these HBV-specific miRNAs may participate in diverse pathways involved in HCC development and progression (Supplementary Table S7). Further studies are warranted to evaluate these miRNAs to aid in understanding the molecular mechanisms underlying miRNA function and to discover potential therapy targets for HCC. Both miRNAs and their target mRNAs could be potential therapeutic targets. Kota et al. (47) have demonstrated that HCC cells expressed a reduced miR-26a and systemic administration of this miRNA results in inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression without toxicity. Because miR-375 and miR-92a are identified from both approaches and likely serve a role in HCC development following chronic HBV infection, future study may focus on their target genes and the mechanisms by which they execute their functions during HBV infection and HBV-positive HCC development.

In conclusion, we have demonstrated that the expression profile of serum miRNAs can serve as novel noninvasive biomarkers for the diagnosis of HBV infection and HBV-positive HCC. Our strategy of using Solexa sequencing followed by qRT-PCR validation provides a successful approach

to identifying serum miRNA profiles as biomarkers for the diagnosis of various diseases.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest was disclosed.

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## Correction: Serum microRNA Profiles Serve as Novel Biomarkers for HBV Infection and Diagnosis of HBV-Positive Hepatocarcinoma

In this article (Cancer Res 2010;70:9798–807), which was published in the December 1, 2010 issue of *Cancer Research* (1), the footnote regarding the equal contribution of the first three authors is incorrect. The footnote should read "L-M. Li, Z-B. Hu, and Z-X. Zhou contributed equally to this work."

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1. Li L-M, Hu Z-B, Zhou Z-X, Chen X, Liu F-Y, Zhang J-F, et al. Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. *Cancer Res* 2010;70:9798–807.

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