Research Paper

Identification of an 88-microRNA signature in whole blood for diagnosis of hepatocellular carcinoma and other chronic liver diseases

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ABSTRACT

Hepatocellular carcinoma (HCC) is a common cancer with very poor survival due to lack of reliable biomarker for early diagnosis. In this study, we investigated microRNA (miRNA) profile of whole blood with a custom microarray containing probes for 1849 miRNA species in a total 213 successive subjects who were divided into a discovery set and a validation set. An 88-miRNA signature was established to diagnose health controls (HC), chronic hepatitis B (CHB), liver cirrhosis (LC) and HCC with 100% accuracy in the discovery set using Fisher discriminant analysis. This diagnostic signature was confirmed in the validation set with accuracy rates of 100%, 95.2%, 93.7% and 98.4% for HC, CHB, LC and HCC patients, respectively. Compared with AFP, the only available non-invasive and routinely used biomarker for diagnosis of HCC, the 88-miRNA signature has much higher accuracy (99.5% vs 76.5%), sensitivity (100% vs 63.8%), and specificity (99.2% vs 84.2%). More importantly, the signature detects small HCCs (<3cm) with 100% (17/17) accuracy while AFP has only 64.7% (11/17). In conclusion, we have identified a powerful and sensitive blood 88-miRNA signature for diagnosing early HCC and other chronic liver diseases (CHB and LC) with a high accuracy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer and the fifth most prevalent malignancy worldwide [1]. Although there are many advances in treatment, HCC patients still have very poor overall survival with 5-year survival rate below 12% [2]. The main reasons for such low survival rate of HCC are asymptomatic and diagnosed at advanced stages due to lack of accurate and non-invasive diagnostic tools for early detection of HCC [3], resulting in missing the best opportunity for curative surgery. In China, more than 401,000 new patients are diagnosed of HCC and more than 371,000 HCC patients

die from this disease every year [4]. Furthermore, nearly 10% of the Chinese population is the carrier of hepatitis B virus (HBV) [5]. Approximate 10% of the patients with chronic hepatitis B (CHB) develop into liver cirrhosis (LC), the leading risk factor for HCC. In addition, some HCCs can directly arise from chronic hepatitis B virus (HBV) infection. Therefore, HCC diagnosis requires differentiation from CHB and LC. Currently, the main non-invasive methods for diagnosis of HCC include ultrasonography and AFP serology. Although serum Alpha-fetoprotein (AFP) has been used for decades as a diagnostic biomarker for HCC, it cannot be used as an independent diagnostic marker because of unsatisfactory sensitivity and specificity. For example, serum AFP level may be elevated in patients with CHB and LC also. Ultrasonography is a useful and non-invasive method for detection and surveillance of HCC, but it does not differentiate well between liver benign and malignant nodules, especially for the small ones (< 2cm) in patients with LC and/or HCC [6]. Therefore, there is an urgent need to identify novel, effective, sensitive, specific and non-invasive biomarkers for early diagnosis of HCC in order to improve the survival of HCC patients.

MicroRNAs (miRNAs) are a class of small (~21 nucleotide) noncoding RNAs that generally negatively regulate the expression of their target genes [7]. Dysregulation of miRNA expression is a common feature in human cancers including hepatocellular carcinoma (HCC) [8-10]. The circulating miRNAs in the plasma, serum or whole blood are considered to be ponderable, stable and noninvasive biomarkers for cancer diagnosis [11-12]. Numerous tumor-derived miRNAs have been reported to be detected in the serum, plasma or blood of cancer patients, which are useful as diagnostic biomarkers for many cancers [13-16]. In 2010, Li L et al. reported the first study in which they first screen miRNAs in two pooled serum samples using Solexa sequencing, and then identified and validated two sets of serum miRNAs for diagnosis of HCC with high accuracies in larger serum sample size by quantitative RT-PCR [8]. Since then, numerous studies on circulating miRNAs (either panel of miRNAs or single miRNA) for diagnosis and prognosis of HCC have been reported [17-19]. However, other than Li's report, so far there are only three diagnostic studies on the circulating miRNA profiling for diagnosis of HCC using high-throughput methods. First, in 2011, Zhou et al employed a microarray to screen 723 miRNAs in 137 plasma samples, established a 7-miRNA panel for diagnosing HCC in 407 plasma samples, and finally validated the panel of miRNAs in 390 samples with diagnostic accuracy of 89% [20]. In 2015, Wen et al applied TLDA Chips to screen 377 miRNAs in 9 plasma samples and identified an 8-miRNA panel as biomarkers for detection of HCC in discovery set (85 samples) and validation set (64 samples) with diagnostic accuracies of 82.3% and 78.0%, respectively [21]. In 2017, Zhu et al used deep sequencing to screen miRNAs in 100 serum samples and identified a 2-miRNA panel for diagnosing HCC with accuracies of 84.2% and 83.6% in training set and validation set, respectively [22]. However, these signatures remain unsatisfactory due to a low diagnostic accuracy of less than 90%. Furthermore, the reliability and feasibility of these signatures remain to be further validated in clinic. In addition, our experience shows that the quantity and quality of RNAs isolated with most commercial kits from serum or plasma is of poor yield and reproducibility, causing inconsistent results even with the same samples (data not published). The latter may explain why serum or plasma miRNAs are difficult to develop as biomarkers in clinical practice.

Recently, individual or set of miRNAs derived from whole blood sample has been reported as new biomarkers for early detection of pancreatic cancer [16, 23, 24], ovarian cancer [25], lung cancer [26-28], and gallbladder cancer [29]. miRNAs sourced from the whole blood including mononuclear cells can be used as diagnostic biomarkers based on the theory that circulating blood cells monitor the patients' physiological and pathological state and respond by altering their transcriptome [30]. The advantages of whole blood miRNA samples are as follows: 1) high miRNA yield [31], 2) less error-prone than the serum or plasma samples, and 3) the whole blood samples contain both tumor-secreted miRNAs and other miRNAs that change following tumor progress, the inflammatory or immunoreactive stage, which yield more comprehensive information than the serum or plasma samples [24, 25]. Other than solid cancers, the whole blood miRNAs can be sourced from distant tissues such as inflammatory foci, neutrophils, monocytes, platelets, and mature red blood cells. Thus, they are more sensitive in inflammation-related cancers such as chronic pancreatitis related pancreatic cancer and HBV related HCC [24]. To our knowledge, there has not been any report on the diagnostic value of whole blood miRNAs in HCC patients to date.

Here, we present a multicenter study on the whole blood miRNA expression profile with a custom microarray in a total of 213 cases consisting of 43 healthy controls (HC), 45 chronic hepatitis B (CHB) patients, 45 liver cirrhosis (LC) patients and 80 HCC patients. In this study, we identified an 88-miRNA signature that accurately diagnose patients with HCC, CHB and LC in a discovery set (150 cases), which was confirmed in a validation set (63 cases).

RESULTS

Clinical characteristics of the patients

To profile miRNA expression in whole blood, we initially collected 150 blood samples as a discovery set for identification of diagnostic signature. After establishment of a diagnostic signature, we obtained another 63 blood samples as a validation set to verify the diagnostic signature. As shown in Table 1, Alanine aminotransferase (ALT), Aspartate transaminase (AST) and Globuline (GLOB) levels are significantly higher in patients with chronic liver diseases including CHB, LC and HCC compared with HCs, while albumin (ALB) is decreased in the patient groups, which indicates a typical liver damage in the patient groups. Among the patients, a significant percentage of HCC patients has higher levels of ALT and AST than those with CHB and

Table 1. Comparison of clinical characteristics of patients and controls

Clinical characteristics	HC (N = 43) n (%)	CHB (N = 45) n (%)	LC (N = 45) n (%)	HCC (N = 80) n (%)	*P* value
Age (years)	42.2 ± 8.7	43.3 ± 12.7	49.8 ± 12.5	49.0 ± 13.0	0.725[a]
≤ 40	18 (41.8)	19 (42.2)	10 (22.2)	50 (62.5)	0.109
> 40	25 (58.1)	26 (57.8)	35 (77.8)	30 (37.5)	
Sex ratio					
male	22 (51.2)	31 (68.8)	34 (75.6)	66 (82.5)	0.217[b]
female	21 (48.8)	14 (31.2)	11 (24.4)	14 (17.5)	
ALT (U/L)	18.5 ± 10.9	104.2 ± 200.7	57.5 ± 62.3	104.0 ± 64.1	<0.001[a]
≤ 40	42 (97.6)	24 (53.3)	22 (48.9)	12 (15.0)	<0.001[b]
> 40	1 (2.4)	21 (46.7)	23 (51.1)	68 (85.0)	
AST (U/L)	17.8 ± 5.6	42.7 ± 38.2	54.4 ± 45.8	77.3 ± 42.2	<0.001[a]
≤45	43 (100.0)	31 (68.9)	22 (48.9)	16 (20.0)	<0.001[b]
>45	0 (0.0)	14 (31.1)	23 (51.1)	64 (80.0)	
ALB (g/L)	39.5 ± 3.1	34.6 ± 3.8	32.7 ± 4.0	32.3 ± 3.4	<0.001[a]
≤ 35	3 (6.9)	31 (68.9)	34 (75.6)	61 (76.3)	<0.001[b]
> 35	40 (93.1)	14 (31.1)	11 (24.4)	19 (23.7)	
GLOB (g/L)	21.2 ± 6.9	31.0 ± 6.1	32.9 ± 5.3	32.5 ± 7.5	<0.001[a]
≤ 35	42 (97.7)	34 (75.6)	29 (64.4)	51 (63.8)	0.001[b]
> 35	1 (2.3)	11 (24.4)	16 (35.6)	29 (36.2)	
HBsAg					
Positive	N/A	45 (100.0)	43 (95.6)	78 (97.5)	0.003
Negative	N/A	0 (0.0)	2 (4.4)	2 (2.5)	
HBV DNA (IU/ml)	N/A	$1.6\times10^5 \pm 5.7\times10^5$	$2.9\times10^6 \pm 8.3\times10^6$	$6.4\times10^5 \pm 2.1\times10^6$	0.009[a]
$< 10^3$	N/A	1 (2.2)	5 (11.1)	6 (7.5)	0.309
$> 10^3$	N/A	44 (97.8)	39 (88.9)	74 (92.5)	
AFP (μg /L)	21.0 ± 7.4	39.1 ± 58.5	49.9 ± 90.5	2106.4 ± 3401.4	<0.001[a]
≤ 25	43 (100.0)	28 (62.2)	33 (73.3)	29 (36.3)	<0.001[b]
> 25	0 (0.0)	10 (37.8)	11 (26.7)	51 (63.7)	

[a]*P* value of one-way ANOVA; [b] Chi-square test; N/A, data is not available

Abbreviation: HC, health control; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ALT, Alanine aminotransferase; AST, Aspartate transaminase; GLOB, Globuline; ALB, albumin; AFP, Alpha-fetoprotein.

LC indicating that these HCC patients already have liver damage. In addition, 97.5% of HCC patients have HBsAg positive, demonstrating that nearly all of HCC patients are infected with HBV virus. Finally, 63.7% of HCC patients have AFP positive, and 37.8% of CHB and 26.7% of LC patients also have AFP positive, suggesting that AFP is not a specific marker for HCC.

MicroRNA expression profiles of whole blood from HCs and patients with CHB, LC and HCC in the discovery set and verification of microarray data by qRT-PCR

In this study, we investigated miRNA expression profiles from whole blood in a total of 213 cases of HC, CHB, LC and HCC subjects. With SAM program and student t test, we found that there are 275 differentially expressed miRNAs with >1.5-fold change between HCs and patients with CHB, LC and HCC (q-value (%) = 0), in the discovery set, 231 of which are up-regulated, and 44 down-regulated in the patients. To validate the microarray results, miR-4508, miR-135a-3p, miR-1273f and miR-92b-3p were examined by qRT-PCR in 40 plasma samples consisting of 10 HC, 10 CHB, 10 LC and 10 HCC subjects randomly selected from the discovery set. Quantitative RT-PCR results showed that the four miRNAs are upregulated in patients with CHB, LC and HCC compared with HCs (Fig. 1), which is consistent with the results obtained by microarray analysis. These results demonstrate the reliability and reproducibility of the microarray data.

Identification of an 88-miRNA diagnostic signature in the discovery set

Upon profiling miRNA expression in whole blood samples, we employed the 275 differentially expressed miRNAs to identify signatures with diagnostic value for HC (30 subjects), CHB (30 subjects), LC (30 subjects), and HCC (60 subjects) in the Discovery set. Fisher discriminant analysis (Stepwise discriminant method) was used to find the best combination of miRNAs that can distinguish the four groups of HC, CHB, LC, and HCC. An 88-miRNA diagnostic signature (Supporting

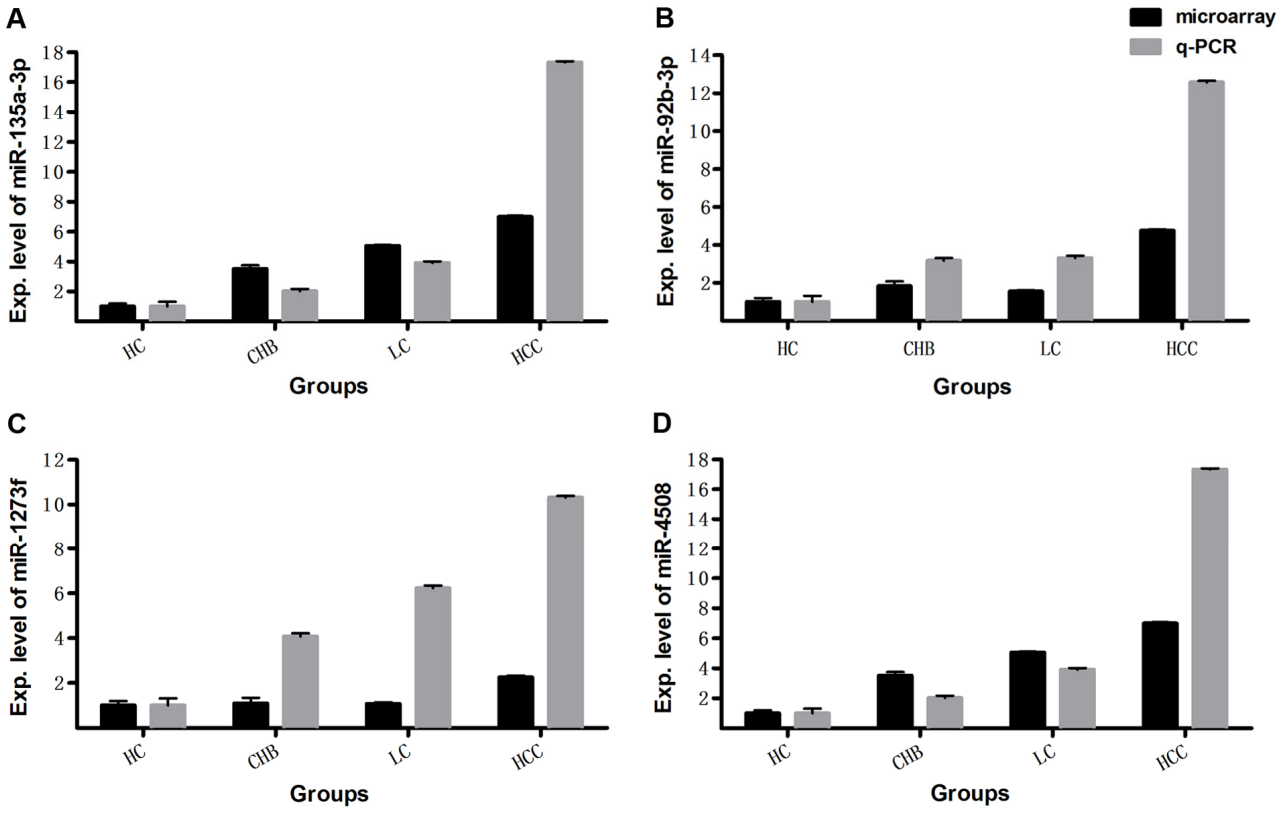


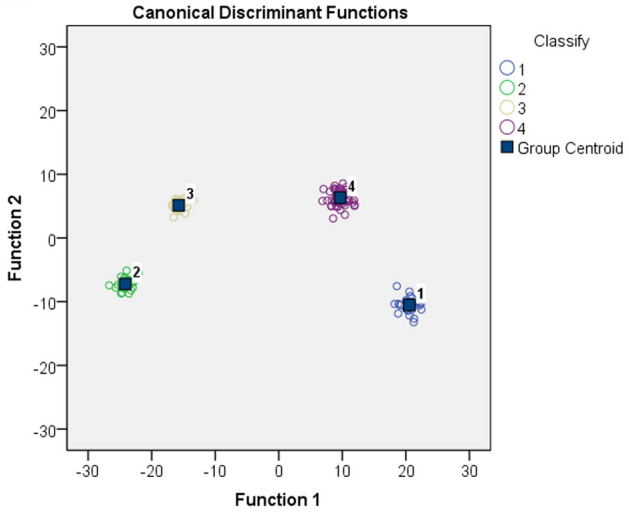
Figure 1. The expression levels of miRNAs detected by microarray were validated with qRT-PCR. The relative expression levels of miR-135a-3p (**A**), miR-92b-3p (**B**), miR-1273f (**C**), miR-4508 (**D**) were examined by qRT-PCR in another 40 whole blood samples consisting of 10 HC, 10 CHB, 10 LC and 10 HCC subjects, and compared with microarray data in the same 40 samples. The qRT-PCR reaction of each sample was performed in triplicate and the mean values were calculated. Relative expression levels are presented with the mean value of qRT-PCR or microarray data in 10 samples of each group.

Table 1) was identified in the discovery set. For diagnosing the four different subjects, four Fisher's discriminant formulas were constructed based on the 88 miRNA expressions: $score_{(i)} = constant_{(i)} + \sum coefficients_{(i)} * miRNA\ expression\ values$. In the formula, (i) represents HC, CHB, LC or HCC. In the four formulas, the same miRNA expression value of each subject multiplies 4 different coefficients for HC, CHB, LC and HCC. In general, HCs have the lowest score of the 88 miRNAs and HCCs have the highest score among the four groups. With the four formulas, four diagnostic scores were calculated for each subject. If the highest score was presented in the formula for HC, the subject was predicted as HC; if the highest score is in the formula for HCC, the subject was predicted as HCC, and all subjects could be predicted in the same manner (Supporting Table 2). Interestingly, the 88-miRNA signature correctly diagnosed all 150 subjects including HCs, CHBs, LCs and HCCs with 100% accuracy (Fig. 2A - 2C).

Verification of the 88-miRNA diagnostic signature in the validation set

To further verify this signature, we collected another 63 blood samples as a validation set to test the diagnostic

A



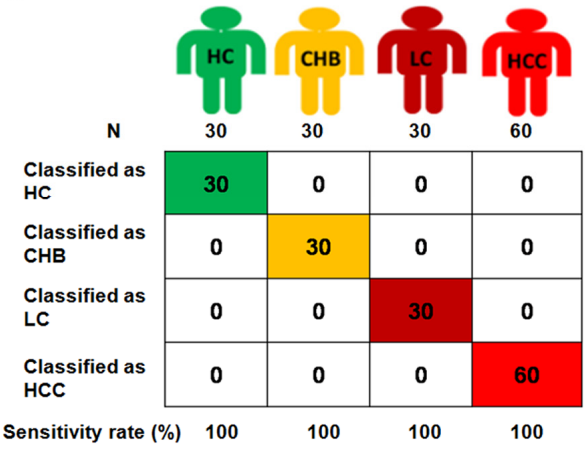
B

Classification Results[a,c]

Classify			Predicted Group Membership				Total
			1	2	3	4	
Original	Count	1	30	0	0	0	30
		2	0	30	0	0	30
		3	0	0	30	0	30
		4	0	0	0	60	60
	%	1	100.0	0	0	0	100
		2	0	100.0	0	0	100
		3	0	0	100.0	0	100
		4	0	0	0	100.0	100
Cross-validated[b]	Count	1	30	0	0	0	30
		2	0	30	0	0	30
		3	0	0	30	0	30
		4	0	0	0	60	60
	%	1	100.0	0	0	0	100
		2	0	100.0	0	0	100
		3	0	0	100.0	0	100
		4	0	0	0	100.0	100

a. 100.0% of original grouped cases correctly classified.
b. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
c. 100.0% of cross-validated grouped cases correctly classified.

C



D

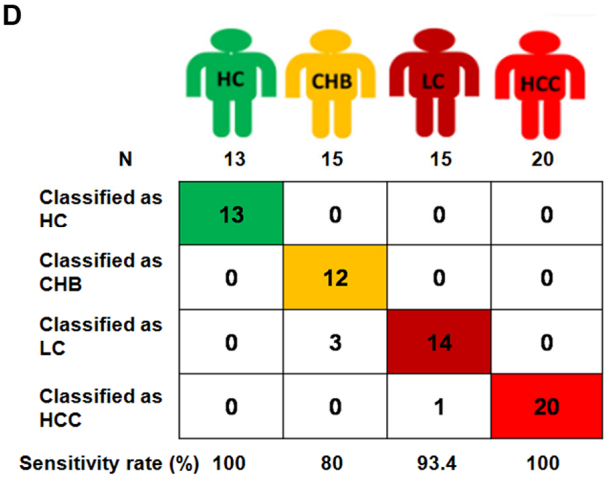


Figure 2. The 4 different groups were classified by 88-miRNA signature in discovery and validation sets. Fisher discriminant analysis (Stepwise discriminant method) of Health control (HC), chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) subjects was performed to establish a 88-miRNA signature in the discovery set. With the 88-miRNA signature, HC, CHB, LC and HCC groups were classified and presented with classification plot (**A**), classification table (**B**), and classification sketch (**C**) in the discovery set. (**D**) The HC, CHB, LC and HCC groups were classified by the signature in validation set and presented with classification sketch.

reproducibility. These samples were detected with the same miRNA microarray. The same four formulas of the 88-miRNA signature obtained from the discovery set were used to calculate the diagnostic score for each subject in the validation set (Supporting Table 3). As expected, the diagnostic accuracy rates are 100% (Sensitivity [Se]: 100%, Specificity [Sp]: 100%), 95.2% (Se: 80.0%, Sp: 100%), 93.7% (Se: 93.3%, Sp: 93.8%) and 98.4% (Se: 100%, Sp: 97.7%) for HC, CHB, LC and HCC, respectively (Fig 2D), which are very similar to those results obtained in the discovery set, especially for HCs and HCCs with 100% sensitivity in both sets. These results indicate that the 88-miRNA signature is a powerful and reproducible diagnostic biomarker for CHB, LC and HCC patients.

The diagnostic value of the 88-miRNA signature is much better than AFP for HCC

In clinical practice, AFP is the only available biomarker routinely used as a non-invasive method for the diagnosis of HCC as a non-invasive method. However, the diagnostic sensitivity of AFP for HCC is only 60-70% [32, 33]. To validate whether the 88-miRNA signature is superior to AFP, we compared both markers by receiver operating characteristic (ROC) analysis. The ROC curves demonstrated that 88-miRNA signature has a much higher diagnostic accuracy for HCC (area under the curve [AUC]: 1.000) than AFP (AUC: 0.728, P<0.001) in discovery set (Fig 3A). This result was further verified in the validation set (signature vs AFP, AUC: 0.988 vs 0.767, P<0.001, Fig 3B).

Table 2. Comparison of HCC diagnostic efficiency of blood 88-microRNA signature and serum AFP on all of 213 subjects

	88-microRNA signature	AFP
Sensitivity % (n/n)	100.0 (80/80)	63.8 (51/80)
Specificity % (n/n)	99.2 (132/133)	84.2 (112/132)
Accuracy % (n/n))	99.5 (212/213)	76.5 (163/213)
Positive predictive value % (n/n)	98.8 (80/81)	70.8 (51/72)
Negative predictive value %(n/n)	100 (132/132)	79.4 (112/141)

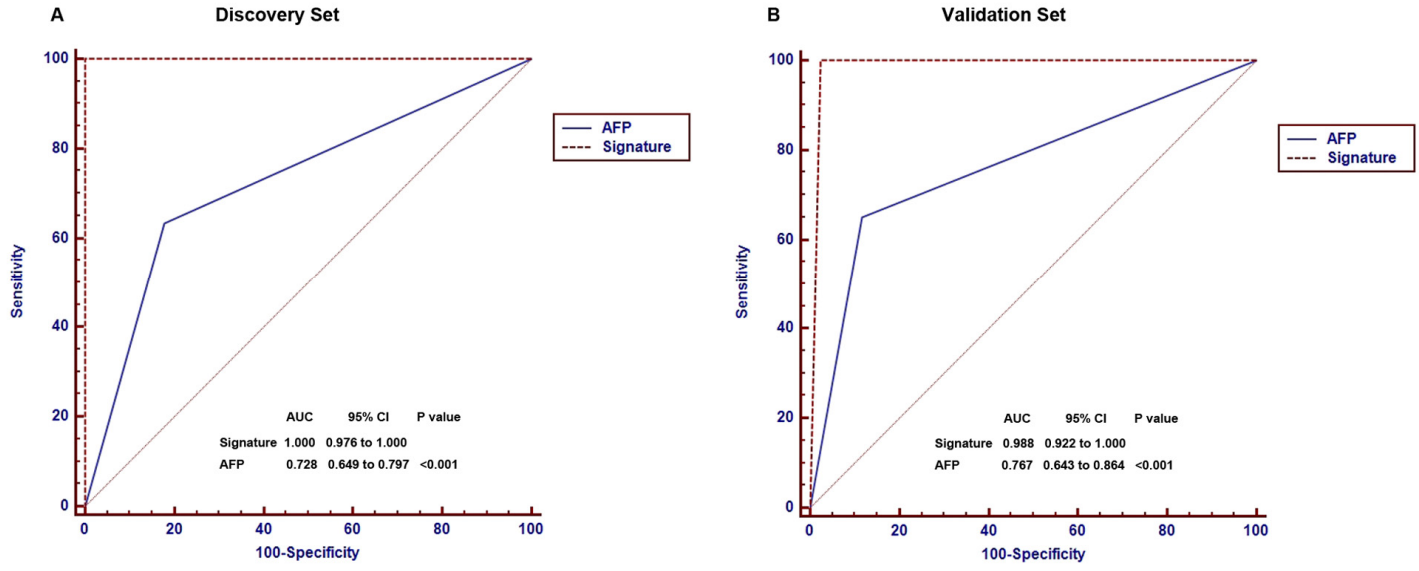


Figure 3. Comparison of diagnostic accuracies of the blood 88-miRNA signature and serum AFP for HCCs and non-HCC subjects in discovery and validation sets. (**A**) The diagnostic accuracies of the blood 88-miRNA signature and serum AFP was compared by Receiver operating characteristic (ROC) analysis in the discovery set. (**B**) The diagnostic accuracies of the blood 88-miRNA signature and serum AFP by ROC analysis in the validation set.

To directly demonstrate that the 88-miRNA signature is superior to AFP for diagnosis of HCC, we compared the two markers in all of the 213 subjects. The result indicates that the 88-miRNA has 99.5% (212/213) accuracy, 100% (80/80) sensitivity and 99.3% (132/133) specificity for diagnosis of HCC, while AFP only has 78.9% (163/213) accuracy, 63.8% (51/80) sensitivity and 86.8% (112/133) specificity (Table 2, Supporting Table 4). These results demonstrate that the 88-miRNA signature is a more powerful, sensitive and reproducible biomarker for HCC than AFP.

More importantly, the 88-miRNA signature correctly diagnosed all of the 17 HCC patients whose tumors are less than 3 cm (median 2.3 cm, ranging from 1.2 to 2.9 cm). In contrast, AFP only correctly determined 64.7 percent (11/17) of the patients with small tumor (Median 2.7, ranging from 1.5 to 2.9; Table 3). These results indicate that the 88-miRNA signature can benefit early diagnosis of HCC.

DISCUSSION

With the advance in high-throughput detection techniques for miRNAs, more and more circulating miRNAs have been found to correlate with cancer diagnosis, progression, prognosis and treatment response, indicating that these miRNAs have great potential for improving diagnosis, prognosis and therapy in cancer patients [34]. In the normal population, the composition of circulating miRNAs most closely correlates with that of liver miRNAs [35], suggesting that under normal conditions liver is the main source for circulating miRNA. Therefore, when lesions (including cancers and HBV infection) occur in the liver, the composition of circulating miRNAs change accordingly, allowing liver diseases to be detected by profiling blood miRNAs.

In this study, we performed a multicenter study on blood miRNA profiles of chronic liver diseases with a

Table 3. Comparison of serum 88-microRNA signature and AFP for diagnosis of small HCC (<3.0 cm).

No	ID	HCC Size (cm)	Signature score				Signature diagnosis	AFP	AFP diagnosis
			Predict value for HC	Predict value for CHB	Predict value for LC	Predict value for HCC		Conc. (ng/mL)	
1	10	1.7	13410	10316	13160	13867	HCC	18.0	No
2	11	1.6	10013	9145	11110	11001	HCC	128.4	HCC
3	16	2.3	400	1686	213	2726	HCC	7.0	No
4	45	2.9	15218	12848	15514	15547	HCC	9307.3	HCC
5	24	2.8	13948	11916	14356	14416	HCC	3294.3	HCC
6	29	1.7	7471	7461	10112	8432	HCC	229.1	HCC
7	31	1.3	5789	4763	5717	6328	HCC	23.8	No
8	36	2.4	7581	6938	7086	8345	HCC	552.2	HCC
9	37	2.9	3207	2819	4524	4726	HCC	1631.7	HCC
10	39	2.9	2854	2364	4123	4346	HCC	6782.6	HCC
11	44	1.5	4264	4920	4055	5037	HCC	41.4	HCC
12	54	2.3	5104	5354	4732	5848	HCC	23.2	No
13	55	2.7	10239	8631	10287	10487	HCC	528.9	HCC
14	73	1.8	8687	7174	8593	8883	HCC	639.5	HCC
15	77	1.2	8816	7287	8779	9035	HCC	9.6	No
16	78	2.7	8442	6805	8130	8775	HCC	337.5	HCC
17	84	2.2	7215	5451	6244	9039	HCC	23.5	No

Abbreviation: Conc., concentration; HC, health control; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; AFP, Alpha-fetoprotein.

custom microarray including 1849 miRNA species, which allowed us identify a diagnostic signature for patients with CHB, LC and HCC. In the miRNA profiling, there are 231 upregulated miRNAs and 44 downregulated ones in patients compared with health controls. The reason for the predominant upregulated miRNAs may be that during the carcinogenesis, more miRNAs are expressed to inhibit the expression of tumor suppressor genes. In contrast, few oncogenes are activated in this manner. It is known that more miRNAs have increased in human cancer [36, 37].

In the discovery phase, an 88-miRNA diagnostic signature was established in a total of 150 available cases, which correctly diagnosed all the 150 cases including HC, CHB, LC and HCC with 100% accuracy. Then we collected another 63 cases consisting of 13 HCs, 15 CHBs, 15 LCs and 20 HCCs from two different medical centers as a validation set. In the validation, the 88-miRNA signature also achieved high diagnostic accuracy: 100% for HC, 95.2% for CHB, 93.7% for LC, and 98.4% for HCC. These results indicate that we for the first time establish a blood 88-miRNA diagnostic signature with high accuracy and reproducibility for chronic liver diseases associated with HBV infection.

Early diagnosis of HCC is critical for enhancing patient survival. Serum AFP was first introduced as diagnostic marker for primary liver cancer in 1964. Since then it has been used for screening and diagnosing HCC worldwide for more than 50 years [38, 39]. However, it has been recognized that single AFP marker has an unsatisfactory sensitivity for detection of HCC because nearly 33 % of HCC patients do not have elevated serum AFP level [40]. The specificity of serum AFP also suffers due to the fact that many patients with benign diseases also have an elevated AFP level. For example, although Zhang et al reported that AFP plus ultrasound surveillance every 6 months in a population with HBV infection significantly reduced HCC mortality by 37% compared with a non-screened population with HBV infection [39], another similar study showed that HBV carriers with periodic AFP screening had no survival benefit compared to those without screening [41]. Therefore, the American Association for the Study of Liver (AASLD) guidelines do not recommend serum AFP surveillance for HCC unless ultrasound is unavailable [6, 42]. Therefore, considerable efforts have been made on finding better serum surrogate markers for HCC than AFP over the last several decades. However, no new surrogate marker for diagnosis of HCC is superior to serum AFP in clinical practice. In this study, we present a blood 88-miRNA signature with 100% and 98.4% diagnostic accuracies for HCC patients in discovery set and validation set, respectively. This is in contrast to 72.8% and 76.7% accuracies for serum AFP in discovery set and validation set. Thus, the blood 88-miRNA signature is a powerful and reproductive surrogate for patients with HCC. Furthermore, the blood 88-miRNA signature can correctly detect 100% (17/17) HCC patients with tumor size less than 3 cm (median: 2.3 cm, ranging: 1.2 - 2.9 cm). In contrast, AFP only diagnose 61.5% (8/13) HCC patients (median: 2.7 cm, ranging: 1.5 -2.9 cm). These results indicate that our blood 88-miRNA signature can lead to early HCC diagnosis of HCC and hence better patient survival. Further studies on small HCC (< 1 cm) detection with the signature are necessary before this blood 88-miRNA signature can be applied in routinely clinical practice. The test also needs to be further verified in larger more HCC patient population and more medical settings.

In early detection of HCC, distinguishing HCC from LC is a big challenge because the nodule configuration of cirrhosis is very similar to that of HCC. Moreover, both HCC and LC patients have elevated AFP level. Worldwide, ultrasound as the main method for HCC surveillance is recommended every 6 months for patients with cirrhosis to increase the early detection rate and survival rate of HCC patients [42]. However, one-fourth of early HCC patients fail to be detected by ultrasonography in early stage HCC patients with cirrhosis [43]. Furthermore, ultrasonography does not distinguish well benign nodules from malignant ones in patients with cirrhosis. In contrast, our blood 88-miRNA signature not only diagnoses HCC with nearly 98.4 - 100% accuracy, but also detects HCC as small as 1.2 cm in diameter. More importantly, this signature can also diagnose liver cirrhosis with 93.7% accuracy. These results suggest that the blood 88-miRNA signature is a potentially powerful biomarker for early screening and diagnosis of HCC.

In summary, we for the first time analyzed the miRNA expression profiles of whole bloods from subjects of HC, CHB, LC and HCC, and established and validated an blood 88-miRNA signature that diagnose CHB, LC and HCC with high accuracies in discovery and validation sets, respectively, which may be a powerful non-invasive biomarker for early diagnosis of HCC patients.

MATERIALS AND METHODS

Patients and tissue samples

A total of 213 cases (containing 43 healthy participants, 45 chronic hepatitis B patients, 45 cirrhosis patients and 80 HBV-related HCC patients) were recruited for this study. Of these subjects, 80 HBV-related HCC patients

collected from the Sun Yat-Sen University Cancer Center, during January 2015 to December 2016, were diagnosed as HCC by pathological examination and did not have any treatments before surgery. The 45 CHB and 45 LC samples were obtained from the Guangzhou Eighth People's Hospital in Guangzhou, where patients with CHB were diagnosed when patients had serum HBsAg positive for more than 6 months, and patients with cirrhosis were diagnosed by liver biopsy. The 43 healthy participants' samples were collected from the Health Examination Center of the Sun Yat-Sen University Cancer Center. All of these samples were sequentially collected and the first 150 samples assigned to a discovery set (containing 30 healthy participants, 30 chronic hepatitis B patients, 30 cirrhosis patients and 60 HBV-related HCC patients) and the second batch of 63 samples into a validation set (containing 13 healthy participants, 15 chronic hepatitis B patients, 15 cirrhosis patients and 20 HBV-related HCC patients). This study was reviewed and approved by the Ethical Committees of Sun Yat-Sen University Cancer Center and Guangzhou Eighth People's Hospital. The written informed consent was obtained from each patient.

RNA extraction

RNA was extracted from 2-3ml of whole blood sample obtained from each patient using the Blood RNA Preservative Tubes and RNA Purification Kit (Norgen Biotek, Thorold, Ontario, Canada) according to the manufacturer's protocol. Briefly, whole blood was mixed well with 1.5 ml of RNA Extraction Buffer A and 1.5 ml of RNA Extraction Buffer B; After incubated in -20 ^{o}C for 10 minutes, the mixture was centrifuged at 4 ^{o}C at 4000 RPM for 30 minutes; After the supernatant was discarded, 570 μL of Resuspension Solution B and 330 μL of 100% Ethanol were added and mixed well; The mixture was added into the Mini Spin column, and then the column was centrifuged at 4 ^{o}C at 3500 RPM for 1 minute; After the column was washed with Wash Solution A for three times, 100 μL of Elution Solution A was added into the column and centrifuged at 4 ^{o}C at 1000 RPM for 2 minutes, followed by 2 minutes at 4500 RPM to elute the RNA sample. Finally, RNA in 100 μL was concentrated to 20 μL, and RNA concentration was measured in an ND-1000 spectrophotometer (NanoDrop Technologies).

Microarray detection

All 1921 human mature miRNAs in the miRBase database (Release 18.0) were used for designing probes for constructing the in-house miRNA microarray and a total of 1849 probes have been successfully designed according to the principle proposed by Wang [44]. The microarray was fabricated in house and hybridized as described by us previously [45, 46]. Briefly, each probe was mixed with printing buffer to a final concentration of 40 μmol/L and printed in duplicate on the cleaned glass slides (75 x 25 mm). The total RNA (1.0 -1.5 μg) was labeled with 100 nmol/L of pCp-Cy5 (Jena Bioscience, Germany) and 15 units of T4 RNA ligase (USB) in a total reaction volume of 20 μL at 16 ^{o}C overnight. Then the mixture of labeled RNA sample and 1x hybridization solution was hybridized onto the microarray for 12 -18 h at 45 ^{o}C. After hybridization, the slides were washed in 1×SSC/1% SDS for 10 min at 45 ^{o}C, followed by sequential washing in 2 cycles of 0.5 ×SSC/0.1% SDS, 2 cycles of 0.2×SSC and 1 cycle of purified water for 1 min at room temperature, respectively, and then dried in a special small centrifuge and scanned using the LuxScan-10K (CapitalBio, China).

Gene expression data extraction

The microarray scanning images were digitized with GenPix Pro 6.0 program, and the raw signal data were extracted, subtracted background and normalized (Quantile normalization) using GPR analysis software (edited in-house). Then we computed the average intensity of the repetitive probes and transformed them into log2 value. The microarray data have been deposited in Gene Expression Omnibus of the National Center for Biotechnology Information (GSE53882).

Quantitative RT-PCR

For qRT-PCR, total RNA (10 ng) was reversely transcribed with TaqMan Assays (Thermo Fisher) including miRNA-specific reverse transcription-primers and MultiScribe Reverse Transcriptase. Quantitative PCR reactions were performed with Universal PCR Master Mix II (TaqMan) on a PRISM 7900HT system (Applied Biosystems) with U6 RNA as the internal control. Each sample was analyzed in triplicate wells, and reactions without cDNA also were included as negative control. The conditions of thermal cycling were as follows: 95 °C at 10 min for a hot start, then 40 cycles at 95 °C for 15 s, 60 °C for 60 s. U6 RNA was used as loading control. The PCR data were first normalized by U6 expression and then by the median expression value of a given microRNA in the corresponding subjects. The relative quantification (RQ) of microRNA expression was presented as $2^{-\Delta\Delta Ct}$.

Statistical analysis

We used student's t test and significance analysis of microarray (SAM) to identify the differentially expressed miRNAs (fold changes >1.5, P<0.001 and

FDR-q <0.05) between healthy subjects and patients' subjects. The differentially expressed miRNAs were used to establish diagnostic miRNA signatures that can distinguish the four groups of HC, CHB, LC, and HCC using Fisher Discriminant Analysis [47] in SPSS Version 20.0 software, and receiver operating characteristics (ROC) analyses were performed to compare the diagnostic accuracies of 88-miRNA signature and AFP in Stata software.

CONFLICTS OF INTEREST

The authors disclose no potential conflicts of interest.

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Supporting Table 1. The expression levels of 88 miRNAs of HC, CHB, LC, and HCC groups in the discovery set.

miRNA	Average value in HC	Average value in CHB	Average value in LC	Average value in HCC	Change fold (Patients/HC)
hsa-let-7a-2-3p	405	4610	2588	662	5.33
hsa-let-7d-5p	2498	21293	15304	8307	5.50
hsa-let-7g-3p	3443	12270	6735	7283	2.50
hsa-miR-103a-3p	8509	3498	3856	3109	0.40
hsa-miR-1247-5p	414	16697	12440	3681	12.03
hsa-miR-1248	405	4879	3602	838	6.17
hsa-miR-126-3p	1306	13331	11640	2144	5.58
hsa-miR-130a-3p	11153	3562	1444	3957	0.27
hsa-miR-132-5p	1780	13949	9531	3249	4.45
hsa-miR-150-5p	1459	45079	37385	10436	17.98
hsa-miR-1537	2417	14258	6090	8928	4.29
hsa-miR-154-3p	2865	31662	22702	5106	6.03
hsa-miR-18b-5p	2035	11121	4390	4276	3.09
hsa-miR-194-3p	1255	8254	3054	1499	3.22
hsa-miR-199a-5p	8029	20463	13660	8748	2.28
hsa-miR-19b-1-5p	8566	3145	1822	3893	0.34
hsa-miR-221-3p	1201	12836	13183	1510	7.19
hsa-miR-23a-5p	625	10196	7067	1163	8.44
hsa-miR-25-5p	592	8282	3755	803	1.63
hsa-miR-2681-5p	481	15051	12339	2372	6.08
hsa-miR-30b-3p	1317	15181	13914	2884	16.95
hsa-miR-30c-1-3p	737	27879	28071	6565	6.69
hsa-miR-30d-3p	1985	11928	12472	4393	11.68
hsa-miR-3145-5p	756	1547	13551	27001	4.11
hsa-miR-3146	703	10723	9364	1159	2.26
hsa-miR-3153	513	14634	14254	3146	8.01
hsa-miR-3162-5p	692	11473	13051	3257	17.54
hsa-miR-3164	1380	9920	10684	2111	11.08
hsa-miR-3184-5p	529	4644	3127	1463	4.47
hsa-miR-3196	412	515	2294	4346	5.57
hsa-miR-34a-3p	928	7841	6115	1635	2.31
hsa-miR-3672	9561	1575	1360	4180	0.50
hsa-miR-3675-3p	4327	4987	4155	14690	4.71
hsa-miR-371b-5p	28252	970	636	15277	0.31
hsa-miR-374c-3p	488	16467	16424	3765	4.30
hsa-miR-378a-5p	11277	33246	36931	13305	2.27
hsa-miR-3908	1094	15529	14541	3373	11.09
hsa-miR-3935	537	14102	9829	3627	2.17
hsa-miR-4284	441	7075	6539	725	8.57
hsa-miR-431-3p	926	26394	21277	10260	15.13

hsa-miR-4322	504	11689	6945	1054	8.76
hsa-miR-4329	3599	37035	39209	11812	6.47
hsa-miR-4418	612	10967	9371	1956	10.69
hsa-miR-4444	673	10185	8032	1895	11.47
hsa-miR-4446-3p	1646	13211	11880	1929	9.99
hsa-miR-4461	524	9177	6991	856	8.21
hsa-miR-4472	492	12258	7935	1497	6.54
hsa-miR-4474-3p	501	16540	17177	2575	8.92
hsa-miR-4476	472	7507	5536	1044	12.12
hsa-miR-4478	779	9952	8633	5058	19.50
hsa-miR-4482-5p	484	939	1150	3148	8.25
hsa-miR-4484	616	5354	6011	1445	10.65
hsa-miR-4495	506	10578	11778	1843	2.24
hsa-miR-449a	524	10721	10284	1371	14.23
hsa-miR-4502	2749	15664	15244	18080	12.98
hsa-miR-4507	691	12581	11140	2335	11.46
hsa-miR-4508	535	15235	10249	5958	6.06
hsa-miR-450a-5p	515	13725	10434	5608	10.51
hsa-miR-450b-5p	465	10215	8594	1541	18.76
hsa-miR-4515	1217	14161	13311	3235	18.03
hsa-miR-4516	1285	24272	24816	6728	12.06
hsa-miR-4522	2999	16253	15327	3679	7.13
hsa-miR-4640-3p	2943	859	715	7431	7.18
hsa-miR-4646-3p	1020	9174	10260	1556	2.49
hsa-miR-4652-3p	31329	1984	1407	10945	0.25
hsa-miR-4677-5p	534	6339	3400	975	6.03
hsa-miR-4706	1296	16999	14658	1500	9.19
hsa-miR-4715-5p	583	10523	8492	816	5.44
hsa-miR-4732-5p	459	7080	5134	810	8.89
hsa-miR-4739	439	3576	4135	1773	8.95
hsa-miR-4750	570	11876	9776	1247	7.54
hsa-miR-4793-5p	931	12926	10451	2639	6.34
hsa-miR-485-5p	2126	13614	14122	2208	11.43
hsa-miR-493-5p	1865	7491	3836	6130	7.92
hsa-miR-5092	620	19903	19179	4477	3.81
hsa-miR-5096	3611	3844	11017	32195	3.20
hsa-miR-526b-5p	526	5882	4993	862	5.50
hsa-miR-541-5p	537	6991	6083	843	9.41
hsa-miR-618	645	30247	22644	4228	7.23
hsa-miR-644b-3p	513	40087	39141	8967	6.37
hsa-miR-662	812	12058	10642	2518	8.51
hsa-miR-767-3p	967	2975	2761	5681	7.33
hsa-miR-767-5p	5182	23261	23596	6076	4.64
hsa-miR-769-5p	5511	41482	40317	14841	8.16
hsa-miR-876-3p	28942	43483	39932	49376	8.64
hsa-miR-876-5p	10690	5379	4989	5926	0.41
hsa-miR-888-3p	3676	3140	1383	5827	2.91
hsa-miR-891b	961	563	7496	7773	5.37

Supporting Table 2. The predicted result of 4 groups with 4 formulas of the 88-microRNA signature in the training set.

Sample	Group	Predict value for HC	Predict value for CHB	Predict value for LC	Predict value for HCC	Sensitivity (%)
1	HC	**4552**	4178	4265	4284	
2	HC	**4504**	4384	4489	4454	
3	HC	**8463**	6548	7801	8143	
4	HC	**9632**	7446	8975	9418	
5	HC	**15401**	12944	14413	15242	
6	HC	**15665**	12893	15566	15032	
7	HC	**19610**	15261	18812	18791	
8	HC	**15262**	12667	15218	14618	
9	HC	**16200**	13130	15917	15578	
10	HC	**17159**	13400	16289	16308	
11	HC	**16710**	13182	16015	16100	
12	HC	**20816**	16000	19704	20421	
13	HC	**16563**	13264	16109	15978	
14	HC	**17101**	13753	16696	16458	
15	HC	**6141**	4746	5464	5803	**100**
16	HC	**8651**	6566	8010	8603	
17	HC	**11657**	9837	10276	10457	
18	HC	**9224**	7219	8768	9187	
19	HC	**9043**	6740	8198	8818	
20	HC	**8516**	6368	7752	8336	
21	HC	**9360**	7399	8877	9290	
22	HC	**9070**	6730	8305	8940	
23	HC	**10033**	7518	9285	9973	
24	HC	**6583**	5702	6537	6314	
25	HC	**8398**	6698	8111	8147	
26	HC	**8290**	7228	7697	7463	
27	HC	**7132**	5165	6150	6827	
28	HC	**13480**	11213	13006	13374	
29	HC	**8834**	6867	8483	8705	
30	HC	**10678**	8326	10122	10613	
31	CHB	8030	**10835**	9766	10648	
32	CHB	10620	**10656**	9645	7998	
33	CHB	8032	**10841**	9768	10648	
34	CHB	3218	**3534**	3074	3498	**100**
35	CHB	7273	**7395**	7206	6591	
36	CHB	3650	**3720**	3228	3715	
37	CHB	3645	**3723**	3226	3716	
38	CHB	3305	**3593**	3156	3592	

39	CHB	5315	**7438**	5931	6374	
40	CHB	2955	**3785**	2485	2844	
41	CHB	4523	**4565**	3962	2986	
42	CHB	6551	**7600**	6839	6490	
43	CHB	6609	**9424**	7196	7883	
44	CHB	5095	**5694**	5006	4309	
45	CHB	4996	**5182**	4901	4293	
46	CHB	7942	**8617**	7814	7439	
47	CHB	2880	**3766**	3213	3014	
48	CHB	3775	**5248**	3665	3795	
49	CHB	1489	**1827**	692	653	
50	CHB	3192	**4205**	2738	3390	
51	CHB	13992	**14170**	14126	11647	
52	CHB	14149	**14313**	14019	11523	
53	CHB	19042	**19168**	18139	14635	
54	CHB	8124	**10642**	9015	8189	
55	CHB	5688	**7949**	7311	5784	
56	CHB	5806	**8147**	7474	5916	
57	CHB	5889	**6766**	6141	5218	
58	CHB	-837	**843**	-732	-1497	
59	CHB	-1603	**-337**	-1957	-2687	
60	CHB	2599	**3261**	2861	3093	
61	LC	3403	3593	**5839**	4162	
62	LC	10686	9267	**12490**	11012	
63	LC	5061	4702	**6919**	6564	
64	LC	4941	4941	**5928**	4727	
65	LC	310	1564	**2233**	402	
66	LC	6524	6218	**7446**	6378	
67	LC	5745	5345	**6544**	5374	
68	LC	4132	4017	**4407**	3038	
69	LC	4175	4072	**5883**	5050	
70	LC	5008	5322	**5516**	4357	
71	LC	4672	4954	**5360**	4108	**100**
72	LC	4421	4257	**6142**	5332	
73	LC	3648	3989	**5148**	3377	
74	LC	3788	3873	**5010**	3684	
75	LC	3307	3824	**3900**	1988	
76	LC	3665	3677	**4493**	2213	
77	LC	5409	5433	**5445**	4569	
78	LC	6663	5681	**9574**	8485	
79	LC	5203	5085	**6413**	4692	
80	LC	-3694	-4278	**-2480**	-5655	
81	LC	-1628	-1021	**591**	-2288	
82	LC	15924	12922	**16188**	16177	

83	LC	14496	12149	**14745**	14630	
84	LC	13017	11159	**13235**	13154	
85	LC	12652	10431	**12780**	12373	
86	LC	6354	7772	**8623**	6385	
87	LC	9104	9612	**11448**	9356	
88	LC	6255	8182	**9009**	6428	
89	LC	8271	7746	**8767**	8268	
90	LC	4638	4647	**5454**	4812	
91	HCC	13410	10316	13160	**13867**	
92	HCC	10013	9145	11110	**11001**	
93	HCC	15483	12301	15487	**16531**	
94	HCC	14641	10974	13978	**15339**	
95	HCC	2706	2199	2831	**2968**	
96	HCC	6307	5400	5988	**6863**	
97	HCC	400	1686	212	**2726**	
98	HCC	4614	3272	2818	**6317**	
99	HCC	11349	9994	11160	**12202**	
100	HCC	7670	6280	6390	**8935**	
101	HCC	7343	5606	6421	**9136**	
102	HCC	7370	6309	7256	**8953**	
103	HCC	13666	11880	14194	**14257**	
104	HCC	13948	11916	14356	**14416**	
105	HCC	15522	13139	15882	**16167**	
106	HCC	15822	13245	16093	**16453**	
107	HCC	24717	18090	22489	**25030**	
108	HCC	20723	16102	19479	**20969**	**100**
109	HCC	7471	7461	10112	**8432**	
110	HCC	6215	7521	6031	**8733**	
111	HCC	5789	4763	5717	**6328**	
112	HCC	7495	7523	7411	**8746**	
113	HCC	6778	5906	6927	**7218**	
114	HCC	4645	5094	4124	**5641**	
115	HCC	7581	6938	7086	**8345**	
116	HCC	3207	2819	4524	**4726**	
117	HCC	2854	2364	4123	**4346**	
118	HCC	6013	6407	5765	**7273**	
119	HCC	5992	5676	5771	**6331**	
120	HCC	6398	5846	6258	**6689**	
121	HCC	4723	4856	4453	**5015**	
122	HCC	4264	4920	4055	**5037**	
123	HCC	10174	9750	10384	**11289**	
124	HCC	5104	5353	4732	**5848**	
125	HCC	10239	8631	10287	**10487**	
126	HCC	10079	10325	9886	**12011**	

127	HCC	11982	10831	12217	**13024**
128	HCC	6397	6641	6407	**7133**
129	HCC	8151	8415	8210	**9563**
130	HCC	9516	8229	9740	**9766**
131	HCC	8488	7150	8559	**8714**
132	HCC	8264	6829	8237	**8698**
133	HCC	8324	6895	8139	**8518**
134	HCC	11699	9787	11747	**12082**
135	HCC	8348	7012	8407	**8620**
136	HCC	7299	6133	7399	**7768**
137	HCC	8693	7287	8682	**8936**
138	HCC	5657	5325	6015	**6270**
139	HCC	8687	7174	8593	**8883**
140	HCC	8777	6907	8285	**9091**
141	HCC	8933	7073	8493	**9272**
142	HCC	8545	6808	8129	**8887**
143	HCC	8816	7287	8779	**9035**
144	HCC	8442	6805	8130	**8775**
145	HCC	8700	6475	7905	**9013**
146	HCC	8200	6571	7845	**8487**
147	HCC	10033	9158	11021	**11130**
148	HCC	394	1682	209	**2721**
149	HCC	4614	3272	2818	**6317**
150	HCC	7215	5451	6244	**9039**

Supporting Table 3. The predicted result of 4 groups with 4 formulas of the 88-microRNA signature in the validation set.

Sample	Group	Predict value for HC	Predict value for CHB	Predict value for LC	Predict value for HCC	Accuracy (%)
1	HC	**7044**	5069	5610	6491	
2	HC	**7066**	4706	5285	6398	
3	HC	**7093**	5076	5629	6538	
4	HC	**7072**	4709	5290	6404	
5	HC	**7226**	5016	5606	6654	
6	HC	**6286**	4154	4524	5603	
7	HC	**5586**	3958	4128	4957	**100**
8	HC	**6262**	4693	5172	5805	
9	HC	**17889**	15450	18354	17390	
10	HC	**7044**	5069	5610	6491	
11	HC	**21047**	16678	20381	19461	
12	HC	**21892**	17031	20741	19931	
13	HC	**19236**	15373	18707	17828	

14	CHB	11605	**15419**	13445	12608	
15	CHB	15076	**17851**	15616	16084	
16	CHB	5069	**7044**	5610	6491	
17	CHB	4706	**7066**	5285	6398	
18	CHB	5076	**7093**	5629	6538	
19	CHB	4709	**7072**	5290	6404	
20	CHB	5016	**7226**	5606	6654	
21	CHB	13557	12197	**14499**	14023	**80**
22	CHB	15860	**16371**	16223	13315	
23	CHB	16839	**17482**	16989	13885	
24	CHB	16148	**16713**	16646	13660	
25	CHB	17134	**17769**	17215	14029	
26	CHB	13663	12150	**14461**	14049	
27	CHB	12435	10845	**12782**	12730	
28	CHB	18529	**19499**	18946	15517	
29	LC	15420	14404	**16653**	15935	
30	LC	16483	15971	**18473**	17256	
31	LC	14931	13816	**16098**	15491	
32	LC	15428	14168	**16677**	16053	
33	LC	16747	15859	**18395**	17363	
34	LC	15533	15132	**17574**	16449	
35	LC	16107	14771	**17534**	16723	
36	LC	13777	15485	**17987**	14604	
37	LC	13741	15445	**17937**	14559	
38	LC	8681	11819	**13062**	9468	
39	LC	12508	13591	**15930**	13976	
40	LC	13387	13848	**15855**	14256	
41	LC	2983	3131	**4284**	3766	
42	LC	16857	14217	**17405**	17684	**93.4**
43	LC	14805	528	**15416**	15253	
44	HCC	15688	12699	**15663**	16662	
45	HCC	17843	15853	**19076**	19571	
46	HCC	19323	16484	**20004**	20806	
47	HCC	18556	15138	**18499**	19666	
48	HCC	18612	15160	**18482**	19556	
49	HCC	18760	15803	**19196**	19919	
50	HCC	20100	16522	**20302**	21222	
51	HCC	19042	15695	**19162**	20184	
52	HCC	18647	15154	**18482**	19675	
53	HCC	19023	15259	**18746**	19791	
54	HCC	19308	15930	**19507**	20620	
55	HCC	17903	14715	**17790**	18620	

56	HCC	17166	14242	17294	**18021**
57	HCC	20114	17136	21035	**21489**
58	HCC	18941	15653	19108	**20107**
59	HCC	20871	17311	21280	**22247**
60	HCC	17506	14956	18059	**18644**
61	HCC	16130	13704	16695	**16736**
62	HCC	15218	12848	15514	**15547**
63	HCC	16059	13553	16513	**16584**

Supporting Table 4. HCC diagnostic efficiency of 88-microRNA and AFP on 213 subjects.

		HCC case N (%)	Non-HCC case N (%)	Total cases
Signature prediction	**Cancer**	80 (100)	1 (0.8)	81
	Non-cancer	0 (0.0)	132 (99.2)	132
AFP prediction	**Cancer**	51 (63.8)	21 (15.8)	72
	Non-cancer	29 (36.2)	112 (84.2)	141
	Total cases	80	133	213