Clinical Significance and Prognostic Value of microRNA Expression Signatures in Hepatocellular Carcinoma

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Abstract

Purpose: MicroRNAs (miRNAs) play important roles in the development and progression of cancer. The aim of this study is to identify miRNA expression signatures in hepatocellular carcinoma and delineate their clinical significance for hepatocellular carcinoma.

Experimental Design: Patients with hepatocellular carcinoma, undergoing hepatectomy were randomly divided into training set (60 patients) and test set (50 patients). Other 56 patients were used as an independent cohort. The miRNA expression levels were detected by microarray and verified by quantitative real-time reverse transcription-PCR (qRT-PCR).

Results: A 30-miRNA signature consisting of 10 downregulated and 20 upregulated miRNAs was established for distinguishing hepatocellular carcinoma from noncancerous liver tissues in the training set with 99.2% accuracy. The classification accuracies of this signature were 97% and 90% in the test set and independent cohort, respectively. The expression level of four miRNAs in the 30-miRNA signature was verified by qRT-PCR in the training set. Twenty miRNAs were then selected to construct prognostic signature in the training set. Of the 20 miRNAs, six were risk factors and 14 were protective factors. A formula based on the 20 miRNAs was built to compute prognostic index. Kaplan–Meier analysis showed that patients with a higher prognostic index had a significantly lower survival than those with a low index. This was verified in the test and independent sets. Multivariate analysis indicated that the 20-miRNA signature was an independent prognostic predictor.

Conclusions: The 30- and 20-miRNA signatures identified in this study should provide new molecular approaches for diagnosis and prognosis of patients with hepatocellular carcinoma and clues for elucidating molecular mechanism of hepatocarcinogenesis. *Clin Cancer Res; 19(17); 4780–91. ©2013 AACR.

Introduction

MicroRNA (miRNA) is a small noncoding RNA of about 22-nt that plays important roles in posttranscriptional gene regulation (1). By base-pairing with the 3’ untranslated regions of target miRNAs, miRNA is capable of degrading target mRNA or downregulating mRNA translation (2).

Since the discovery of the first miRNA lin-4 in 1993 (3, 4), thousands of miRNAs have been identified in human, animals, plants, and viruses by molecular cloning, sequencing, and computational approaches (5, 6). miRNAs are now widely recognized to play important roles in the control of developmental and physiologic processes, especially in developmental timing, cell death, cell proliferation, hematopoiesis, and patterning of the nervous system (7). During cancer development and progression, miRNA can function as tumor suppressor or oncogene (8–11). As such, they are often referred to as Oncomirs (11).

Many studies indicate that miRNA expression signatures or profiles can serve as diagnosis and prognosis predictors for various tumors (12–15). miRNA profiles have been shown to be more accurate for classification of cancers compared with mRNA profiles (14). Several studies on miRNA expression profiles in hepatocellular carcinomas revealed the extensive correlations between miRNA expression and hepatocellular carcinoma development, progression, and therapy (16, 17). However, only a limited number of miRNA species were analyzed in these studies due to the small number of known miRNA available at that time.

In this study, we analyzed the miRNA expression profiles in 166 patients with hepatocellular carcinoma from South
Both hepatocellular carcinoma and corresponding adjacent noncancerous liver (more than 2 cm away from the hepatocellular carcinoma) were sampled. Fresh tissues were immediately immersed in RNA later (Ambion, Inc.) after surgical resection, stored at 4°C overnight, and then frozen at −80°C until use. Total RNA was extracted using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions.

This study was reviewed and approved by the Committee for the Conduct of Human Research of the Sun Yat-Sen University Cancer Center. Written informed consent was obtained from each patient with hepatocellular carcinoma. None of the patients had received any other therapies such as chemoembolization or chemotherapy before surgery.

miRNA microarray fabrication, RNA labeling, and hybridization

Microarray fabrication was carried out as described by Wang and colleagues (18) and probe design and RNA labeling were conducted according to published protocols with minor modifications (19). Briefly, all of the human miRNAs (release 12) in the miRBase (20) were used to design the probes. With the principles described by Wang and colleagues (19), 683 probes for miRNAs were successfully designed for the microarray. For fabrication of microarray, the probes (40 μmol/L final concentration) mixed with printing buffer were printed onto slides in duplicate using SmartArrayer 136 printer (CapitalBio Inc). The quality of microarray was tested using 2 control RNA samples to do hybridization for every batch of microarrays and the correlation among the different microarray batches was analyzed. The correlation analysis showed that the correlation coefficient was more than 95%, indicating that the differences between the batches of microarrays were in the acceptable range. During the labeling reaction, 2.5 μg of total RNA was used with 100 nmol/L of pCp-DY647 or pCp-DY547 (Dharmacon) and 15 units of T4 RNA ligase (USB) in a total reaction volume of 20 μL at 16°C overnight. Labeled RNAs from paired hepatocellular carcinoma and noncancerous liver were mixed and hybridized to the array with a 2 × hybridization solution (final concentration: 5 × Denhart’s solution, 0.5% SDS, and 5 × SSC) in a Hybridization Chamber (Corning Inc.) at 46°C for 12 to 16 hours. After washing, microarray was scanned with a LuxScan 10 K Microarray Scanner (CapitalBio Inc.) at PMT 800 to 900. Scanned images were gridded using the GenPix Pro 6.0 software (Axon Instruments).

Real-time quantitative reverse transcription-PCR

The reverse transcription (RT) was conducted with 2 μg of total RNA isolated from hepatocellular carcinoma or corresponding noncancerous liver tissues, 5 nmol/L of Bulge-Loop miRNA RT specific primers (Ribobio Co.), 0.2 mmol/L dNTP, 40 U RNase inhibitor, 200 U M-MLV reverse transcriptase (Promega) in a 50 μL volume at 42°C for 60 minutes. The quantitative PCR reaction was carried out in a 20 μL volume with 2 μL of RT products, 2 μL of Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen,
USA), and 500 nmol/L each of Bulge-Loop miRNA forward specific primer and universal reverse primer in an Applied Biosystems PRISM 7900HT instrument. The PCR reaction cycle was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, and a dissociation stage. U6 RNA was detected by qRT-PCR as an internal control.

Data process and statistical analyses

Microarray raw data preprocessing included local background subtraction, averaging of intensities of duplicated probes, quantile normalization across multiple arrays, log 2 transformation of expression levels. Only the miRNAs with expression in more than 15% of samples were subjected to further statistical analysis. This normalized microarray data have been deposited into the Gene Expression Omnibus Public Database at the National Center for Biotechnology Information, and the accession number is GSE31384.

Student t test and significance analysis of microarray (SAM) were used to analyze the differential expression of miRNA detected by microarray. Hierarchical clustering analysis (HCL) was conducted using MultiExperiment Viewer version 4.2 (21). Prediction analysis of microarrays (PAM; ref. 22) was used to evaluate the prediction capacity of miRNA signature to classify hepatocellular carcinoma and noncancerous liver samples.

2−ΔΔCt was used to represent expression changes of miRNA between hepatocellular carcinoma and matched noncancerous liver detected by qRT-PCR. Paired Student t test was conducted to analyze the differential miRNA expression levels between tumors and noncancerous liver tissues. The survival risk prediction of patients was done with BRB ArrayTools v.3.7.1 (http://linus.nci.nih.gov/BRB-ArrayTools.html). With GraphPad Prism 5 software (www.graphpad.com), the log-rank test and Kaplan–Meier survival analysis were used to compare the survivals of patients in different risk groups. Cox proportional hazards regression was used to evaluate the HR of miRNA signature and clinical variables for patient survival. All other statistical analyses were conducted using the SPSS 16.0 (SPSS Inc.).

### Table 1. Clinical characteristics of patients with hepatocellular carcinoma in the training, test, and independent sets

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Training set, n (%)</th>
<th>Test set, n (%)</th>
<th>Independent set, n (%)</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>22 (37)</td>
<td>20 (40)</td>
<td>29 (52)</td>
<td>0.232</td>
</tr>
<tr>
<td>&lt;50</td>
<td>38 (63)</td>
<td>30 (60)</td>
<td>27 (48)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td>0.381</td>
</tr>
<tr>
<td>Male</td>
<td>48 (80)</td>
<td>43 (86)</td>
<td>50 (89)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12 (20)</td>
<td>7 (14)</td>
<td>6 (11)</td>
<td></td>
</tr>
<tr>
<td>Pathology grade</td>
<td></td>
<td></td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>I</td>
<td>6 (10)</td>
<td>4 (8)</td>
<td>13 (23)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>32 (53)</td>
<td>39 (78)</td>
<td>32 (57)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>22 (37)</td>
<td>7 (14)</td>
<td>11 (20)</td>
<td></td>
</tr>
<tr>
<td>HBV-DNA Positive</td>
<td>25 (42)</td>
<td>23 (46)</td>
<td>22 (39)</td>
<td>0.8</td>
</tr>
<tr>
<td>HBV-DNA Negative</td>
<td>35 (58)</td>
<td>27 (54)</td>
<td>34 (61)</td>
<td></td>
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<tr>
<td>HBsAg Positive</td>
<td>48 (80)</td>
<td>46 (92)</td>
<td>52 (93)</td>
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<tr>
<td>HBsAg Negative</td>
<td>12 (20)</td>
<td>4 (8)</td>
<td>4 (7)</td>
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<tr>
<td>AFP (μg/L) ≥400</td>
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<td>25 (50)</td>
<td>20 (36)</td>
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<td>25 (50)</td>
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<tr>
<td>Cirrhosis Yes</td>
<td>47 (78)</td>
<td>38 (76)</td>
<td>54 (96)</td>
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</tr>
<tr>
<td>No</td>
<td>13 (22)</td>
<td>12 (24)</td>
<td>2 (4)</td>
<td></td>
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<td>12 (20)</td>
<td>6 (12)</td>
<td>8 (14)</td>
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<tr>
<td>No</td>
<td>40 (80)</td>
<td>44 (88)</td>
<td>48 (86)</td>
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<tr>
<td>Tumor number ≥2</td>
<td>15 (25)</td>
<td>16 (32)</td>
<td>17 (30)</td>
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<td>&lt;1</td>
<td>45 (75)</td>
<td>34 (68)</td>
<td>39 (70)</td>
<td></td>
</tr>
<tr>
<td>Main size ≥5 cm</td>
<td>46 (77)</td>
<td>39 (78)</td>
<td>43 (77)</td>
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<tr>
<td>&lt;5 cm</td>
<td>14 (23)</td>
<td>11 (22)</td>
<td>13 (23)</td>
<td></td>
</tr>
<tr>
<td>Tumor encapsulation None</td>
<td>21 (35)</td>
<td>11 (22)</td>
<td>11 (20)</td>
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<tr>
<td>Incomplete</td>
<td>18 (30)</td>
<td>21 (42)</td>
<td>18 (32)</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>21 (35)</td>
<td>18 (36)</td>
<td>27 (48)</td>
<td></td>
</tr>
<tr>
<td>TNM I</td>
<td>17 (28)</td>
<td>13 (26)</td>
<td>30 (54)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>II</td>
<td>23 (38)</td>
<td>20 (40)</td>
<td>5 (9)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>20 (33)</td>
<td>17 (34)</td>
<td>21 (37)</td>
<td></td>
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<tr>
<td>BCLC Stage 0-A</td>
<td>36 (60)</td>
<td>31 (62)</td>
<td>33 (59)</td>
<td>0.648</td>
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<tr>
<td>B</td>
<td>12 (20)</td>
<td>13 (26)</td>
<td>16 (29)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>12 (20)</td>
<td>6 (12)</td>
<td>7 (12)</td>
<td></td>
</tr>
<tr>
<td>Postsurgical metastasis Yes</td>
<td>8 (13)</td>
<td>6 (12)</td>
<td>5 (9)</td>
<td>0.787</td>
</tr>
</tbody>
</table>

(Continued on the following column)
Results

Identification of a 30-miRNA signature to discriminate hepatocellular carcinoma from corresponding noncancerous liver tissues

miRNA expression profiles of 110 pairs of hepatocellular carcinomas and noncancerous liver tissues in the training and test sets were investigated using a custom miRNA microarray. In the training set, SAM analysis (false discovery rate (FDR) was set to 0) revealed that there were 334 miRNAs with differential expressions between hepatocellular carcinoma and noncancerous liver tissue. Of these miRNAs, 174 were upregulated and 160 were downregulated in hepatocellular carcinoma. To identify a signature to distinguish between hepatocellular carcinoma and noncancerous liver, miRNAs with highest fold changes and smallest P values were selected from the 334 miRNAs in the training set using SAM program (FDR = 0) and paired Student t test. A 30-miRNA signature was developed by class prediction and clustering, which reached the maximum correct classification rate (99.2%) for hepatocellular carcinoma and noncancerous liver (Fig. 1A). Out of the 30 miRNAs (Table 2), 10 were downregulated and 20 upregulated in

![Figure 1](image-url). Hierarchical clustering of hepatocellular carcinoma samples in the training and test sets. A, hierarchical clustering of 60 hepatocellular carcinoma samples (blue bars at the top left) and 60 matched nontumor livers (yellow bars at the top right) in the training set by the 30-miRNA signature; one hepatocellular carcinoma was misclassified into nontumor group and 2 nontumor liver into hepatocellular carcinoma group; each row represents the expression level of an individual miRNA and each column represents an individual tissue sample. Pseudo-colors indicate expression levels from low to high (green to red). The color scale at bottom denotes the gene expression levels from -3 to 3 in log base 2 units. B, hierarchical clustering of 50 hepatocellular carcinoma samples (blue bars) and 50 matched nontumor livers (yellow bars) in the test set by the same signature.
hepatocellular carcinoma. The effectiveness of the 30-miRNA signature was verified in 50 paired hepatocellular carcinoma and noncancerous liver samples of the test set (Fig. 1B) and 56 paired samples of the independent cohort (Supplementary Fig. S1A). To further test the effectiveness of the 30-miRNA signature, PAM analysis was conducted in the test set and the independent cohort (Supplementary Fig. S1B and S1C). The Predicted Classes and Predicted Posterior Probabilities were listed in Supplementary Table S2. In the test set, the accuracy, sensitivity, and specificity for distinguishing between hepatocellular carcinoma and noncancerous liver were 97%, 100%, and 94%, respectively; in the independent cohort, those were 90%, 94%, and 88%, respectively. This result suggested that the 30-miRNA signature for distinguishing between hepatocellular carcinoma and noncancerous liver was robust.

In addition, we analyzed the relationship of miRNA expression with cirrhosis, pathologic grade, and clinical stage in whole cohort and found some differential expression of miRNAs between these characteristics using the criteria of fold change > 1.5 and $P < 0.05$ (Supplementary Table S3).

**qRT-PCR validation of miRNA expression levels detected by microarray**

To validate the miRNA expression level detected by microarray, we carried out qRT-PCR for 2 upregulated miRNAs (mir-17 and mir-21) and 2 downregulated ones (mir-269-5p and mir-625) in 60 paired hepatocellular carcinoma and noncancerous liver samples from the training set. In the qRT-PCR assay, U6 gene was used as an endogenous control. The results showed that the expression levels of the four miRNAs detected by qRT-PCR were significantly different between hepatocellular carcinomas and noncancerous liver tissues as indicated by paired student $t$ test (all $P < 0.05$, Fig. 2A), and strongly correlated with the microarray data (Fig. 2B). These results show that miRNA levels detected by microarray are reliable and can be used for the further analysis.
Identification of 20 miRNAs associated with survival in the training set

The microarray raw data in the training set were normalized and transformed into a log 2 ratio of hepatocellular carcinoma to noncancerous liver by BRB-ArrayTools and then miRNAs with expression in less than 15% of samples and change in expression less than 1.5-fold were filtered (23, 24). To identify a miRNA signature correlated with patients' survival, the relationship between miRNA expression levels and patients' survival was assessed in the training set by fitting Cox proportional hazards models using BRB-ArrayTools software and miRNAs were chosen by the criteria of \( P < 0.1 \). Then different combinations of miRNAs were tested for predicting hepatocellular carcinoma survival. In this way, a best combination (20 miRNA) was selected to achieve the best prediction of survival of patients with hepatocellular carcinoma. A simple formula for computing prognostic index was established from the 20 miRNAs:

\[
\sum w_i x_i = \text{PI}
\]

where \( w_i \) and \( x_i \) are the weight and logged gene expression level for the \( i \)-th gene, and the weight of each miRNA is shown in Table 3. Of the 20 miRNAs, 6 miRNAs were risk factors that were defined as those with hazard ratio (HR) for death more than one, and the other 14 miRNAs were protective factors that were defined as those with HR for death less than one (Table 3). With this formula, each patient had a prognostic index and the median of prognostic index for all patients in training set was \(-0.090\). Patients were assigned to a high-risk group if their prognostic index was more than \(-0.090\) or a low-risk group if their prognostic index was equal to or less than \(-0.090\).

Correlation between the 20-miRNA signature and patient survival in the training set

Using the 50th percentile of the 20-miRNA prognostic index as a cut-off point (\(-0.090\)), 60 patients in the training set were separated into a high-risk (high index) subgroup or a low-risk (low index) subgroup according to their
prognostic index. There were 30 patients in high-risk group with a 3-year survival rate of 35.3% and 30 in the low-risk group with a 3-year survival rate of 92.6%. Kaplan–Meier survival analysis of the patients in the two subgroups revealed that both OS rate and DFS rate in the high-risk group were significantly lower than those in the low-risk group (P < 0.001 and P < 0.001, respectively, see Fig. 3A).

Validation of the 20-miRNA signatures for survival prediction in the test set

To validate the 20-miRNA signature for predicting survival of patients with hepatocellular carcinoma, 50 patients in the test set were classified into a high-risk subgroup and a low-risk subgroup on the basis of the same Cox proportional hazards model and cut-off point, which were obtained from the training set. Kaplan–Meier survival analysis on the patients with high-risk (n = 23) and low-risk (n = 27) also was conducted as described for the training set. As expected, the OS and DFS rate of the patients with high-risk were markedly poorer than those of patients with low-risk (P = 0.033, P = 0.039, respectively, Fig. 3B).

The 20-miRNA signature for survival prediction was further verified in an independent cohort

Furthermore, to validate the 20-miRNA signatures association with hepatocellular carcinoma survival, another 56 patients with hepatocellular carcinoma received the hepatectomy in the same hospital during 2001 to 2003 were used as an independent cohort and detected by the miRNA microarray. Data processing was conducted as described above. The 56 patients were split into a high-risk subgroup (n = 29) and a low-risk subgroup (n = 27) according to the same Cox model and cut-off point. Kaplan–Meier survival analysis showed similar results to those in the training set and test set. The OS and DFS of high-risk group were significantly worse than those of the low-risk group (P = 0.033 and P = 0.040, respectively, Fig. 3C).

To directly show the relationship between the 20-miRNA prognostic index and survival, we plotted the prognostic index and survival status of all patients in training and test sets (Supplementary Fig. S2). In this plot, patients with low-risk scores had much less deaths than those with high-risk score. Likewise, similar results were found in the training set, test set, and independent cohort (Supplementary Fig. S3A–S3C, respectively). All of the analytic data pertaining to survival indicate that the 20-miRNA signature is robustly correlated with hepatocellular carcinoma patient survival.

Univariate and multivariate Cox regression analysis of the 20-miRNA signature and clinical variables

To further verify whether the signature was an independent prognostic factor, the signature and clinical variables in all of 166 patients with hepatocellular carcinoma were analyzed by Cox regression model. First, the univariate Cox regression revealed that the 20-miRNA signature and 7 clinical variables were significant predictors. Then, the multivariate Cox regression showed that the 20-miRNA signature (P < 0.001) and hepatitis B e Antigen (HBeAg: ACGT TGTCCGTGCTG
\begin{table}[h]
\centering
\small
\begin{tabular}{c|p{1cm}|rr|l}
No. & miRNA & Weight & HR (95% CI) & P* & Putative function \\
\hline
1 & hsa-miR-708 & 0.13 & 7.56 (1.52–37.57) & 0.013 & High-risk \\
2 & hsa-miR-34c-3p & 0.08 & 5.21 (1.67–16.34) & 0.005 & High-risk \\
3 & hsa-miR-584 & 0.03 & 4.31 (1.29–14.40) & 0.018 & High-risk \\
4 & hsa-miR-431* & 0.15 & 3.84 (1.97–7.46) & <0.001 & High-risk \\
5 & hsa-miR-744 & 0.12 & 1.74 (1.01–2.98) & 0.045 & High-risk \\
6 & hsa-miR-141 & 0.05 & 1.42 (1.10–1.83) & 0.007 & High-risk \\
7 & hsa-let-7d & –0.24 & 0.28 (0.10–0.83) & 0.022 & Protective \\
8 & hsa-miR-15a & –0.24 & 0.25 (0.08–0.80) & 0.02 & Protective \\
9 & hsa-miR-142-3p & –0.17 & 0.23 (0.05–1.00) & 0.051 & Protective \\
10 & hsa-miR-10b & –0.13 & 0.21 (0.04–1.03) & 0.054 & Protective \\
11 & hsa-let-7e & –0.25 & 0.19 (0.05–0.65) & 0.008 & Protective \\
12 & hsa-miR-28-3p & –0.14 & 0.16 (0.03–0.80) & 0.026 & Protective \\
13 & hsa-miR-193b & –0.15 & 0.16 (0.03–0.82) & 0.028 & Protective \\
14 & hsa-miR-101 & –0.22 & 0.16 (0.03–0.92) & 0.04 & Protective \\
15 & hsa-miR-451 & –0.21 & 0.15 (0.02–1.16) & 0.068 & Protective \\
16 & hsa-miR-142-5p & –0.16 & 0.13 (0.02–0.96) & 0.045 & Protective \\
17 & hsa-miR-26b & –0.24 & 0.11 (0.02–0.62) & 0.013 & Protective \\
18 & hsa-miR-497 & –0.21 & 0.1 (0.02–0.56) & 0.009 & Protective \\
19 & hsa-miR-29c & –0.36 & 0.09 (0.02–0.51) & 0.007 & Protective \\
20 & hsa-miR-140-3p & –0.17 & 0.07 (0.01–0.37) & 0.002 & Protective \\
\end{tabular}
\caption{miRNAs associated with overall survival of patients with hepatocellular carcinoma in the training set}
\end{table}

*aCox proportional hazard regression analysis.
were independent prognostic factors (Table 4). Cox regression analysis on the score of the 20-miRNA signature indicated that the patients with high-risk had a significant higher HR for tumor-related death (HR, 2.75) compared with those with low-risk. In addition, we conducted univariate and multivariate Cox regression analysis on the patients in the 3 sets separately (Supplementary Table S4) and combination of 2 sets (Supplementary Table S5).

Figure 3. Kaplan–Meier curve analysis of (OS) and relapse-free survival (OFS) in patients with hepatocellular carcinoma with high- or low-risk according to the 20-miRNA signature score. A, OS and OFS of patients with high- or low-risk score in the training set. B, OS and OFS of patients with high- or low-risk score in the testing data set. C, OS, and OFS of patients with high- or low-risk score in the independent cohort.

\( P = 0.008 \) were independent prognostic factors (Table 4).
The results were generally consistent with those in the whole cohort.

Discussion

In this study, we fabricated a custom microarray containing 683 miRNA probes to detect miRNA profiles in patients with hepatocellular carcinoma from Southern China. Although there had been some reported miRNA-profiling studies on hepatocellular carcinoma, the total number of human miRNA species in those studies was very limited (about 300 at most; refs. 25, 26). This is the first study in which a microarray consisting of 683 miRNAs has been used in profiling 166 hepatocellular carcinomas. With this microarray, a 30-miRNA signature for discriminating hepatocellular carcinomas from noncancerous liver samples was established in the training set and validated in the test and independent set. This result shows that the signature identified from the training set is highly reproducible in the test and independent sets despite some heterogeneity in the latter set.

Reviewing the articles on miRNA profiling in hepatocellular carcinoma, in 2006, Murakami and colleagues conducted the first miRNA expression profiling study in 25 Japanese patients with hepatocellular carcinoma with a microarray containing probes corresponding to 180 mature miRNAs and 206 precursor miRNAs (27). They identified 8-miRNA signature (7 mature miRNAs and 1 precursor miRNA) that could distinguish hepatocellular carcinoma from nontumor liver with a 97.8% (45/46) of accuracy (27). In this 8-miRNA signature, only one miRNA (miR-18) is present in our 30-miRNA signature. Another Japanese study reported that 23 miRNAs were differentially expressed between 26 pairs of hepatocellular carcinoma and liver tissues with chronic hepatitis B or C, which represents 70.8% of accuracy for distinguishing hepatocellular carcinoma from the corresponding chronic hepatitis B ($n = 12$) and 82.1% for hepatocellular carcinoma from chronic hepatitis C ($n = 14$; ref. 26). Of the 23 miRNAs, only 3 miRNAs (miR-223, miR-21, and miR-221) are present in our 30-miRNA signature (26). Possible reasons for the miRNA discrepancy between our signature and Japanese group’s signatures include different number of miRNAs (683 versus 180 and 188) in the different microarrays, the different pathogens [hepatitis B virus (HBV) vs. HBV and hepatitis C virus (HCV)] and/or genetic variations between Japanese and Chinese patients.

In 2008, Jiang and colleagues revealed 16 miRNAs differentially expressed between 28 pairs of hepatocellular

### Table 4. Univariate and multivariate analysis of features associated with overall survival

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate Cox regression analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-miRNA signature</td>
<td>3.56 (2.15–5.87)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age</td>
<td>0.85 (0.53–1.35)</td>
<td>0.494</td>
</tr>
<tr>
<td>Gender (M vs. F)</td>
<td>1.2 (0.60–2.41)</td>
<td>0.611</td>
</tr>
<tr>
<td>Grade (I, II, III)</td>
<td>1.5 (1.02–2.21)</td>
<td>0.038</td>
</tr>
<tr>
<td>HBV-DNA (positive vs negative)</td>
<td>1.46 (0.92–2.32)</td>
<td>0.11</td>
</tr>
<tr>
<td>HBsAg (positive vs. negative)</td>
<td>1.33 (0.64–2.77)</td>
<td>0.453</td>
</tr>
<tr>
<td>HBeAg (positive vs. negative)</td>
<td>2.03 (1.19–3.48)</td>
<td>0.01</td>
</tr>
<tr>
<td>AFP ($\geq 400$ µg/L vs. $&lt; 400$ µg/L)</td>
<td>1.38 (0.87–2.18)</td>
<td>0.175</td>
</tr>
<tr>
<td>Cirrhosis (yes vs. no)</td>
<td>2.07 (0.95–4.51)</td>
<td>0.068</td>
</tr>
<tr>
<td>Cancer Embolus (yes vs. no)</td>
<td>2.43 (1.44–4.11)</td>
<td>0.001</td>
</tr>
<tr>
<td>Tumor Number (1 vs. $\geq 2$)</td>
<td>2.26 (1.41–3.64)</td>
<td>0.001</td>
</tr>
<tr>
<td>Main size ($\geq 5$ cm vs. $&lt; 5$ cm)</td>
<td>2.92 (1.45–5.87)</td>
<td>0.003</td>
</tr>
<tr>
<td>Tumor encapsulation (complete, incomplete, none)</td>
<td>0.64 (0.48–0.85)</td>
<td>0.002</td>
</tr>
<tr>
<td>TNM stage (I, II, III)</td>
<td>1.87 (1.41–2.50)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Multivariate Cox regression analysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-miRNA signature (high score vs. low score)</td>
<td>2.75 (1.58–4.79)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Grade (I, II, III)</td>
<td>1.03 (0.66–1.61)</td>
<td>0.887</td>
</tr>
<tr>
<td>HBsAg (positive vs. negative)</td>
<td>2.3 (1.29–4.10)</td>
<td>0.005</td>
</tr>
<tr>
<td>Cancer embolus (yes vs. no)</td>
<td>1.74 (0.96–3.13)</td>
<td>0.067</td>
</tr>
<tr>
<td>Tumor Number (1 vs. $\geq 2$)</td>
<td>1.39 (0.76–2.53)</td>
<td>0.281</td>
</tr>
<tr>
<td>Main size ($\geq 5$ cm vs. $&lt; 5$ cm)</td>
<td>1.93 (0.93–4.02)</td>
<td>0.079</td>
</tr>
<tr>
<td>Tumor encapsulation (complete, incomplete, none)</td>
<td>0.94 (0.67–1.31)</td>
<td>0.71</td>
</tr>
<tr>
<td>TNM stage (I, II, III)</td>
<td>1.43 (0.96–2.14)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Abbreviations: AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

*Analysis was conducted on the entire cohort ($n = 166$).

*95% CI.
carcinoma and adjacent benign tissues from the United States using qRT-PCR for 196 mature miRNAs (28). With the 16-miRNA signature for distinguishing hepatocellular carcinoma from noncancerous liver, the correct classification rate was 96.4% (54/56). Out of the 16 miRNAs, 5 miRNAs were present in our 30 miRNAs. In the same year, Li and colleagues found that 69 miRNAs (5 of them were predicted but later proven to be false miRNAs) showed significantly different expression in 78 pairs of hepatocellular carcinoma and corresponding noncancerous liver tissues from Eastern China and 89.7% of the samples could be correctly classified by the 69-miRNA signature (29). Interestingly, 11 miRNAs (hsa-miR-103, hsa-miR-106b, hsa-miR-130b, hsa-miR-18a, hsa-miR-20a, hsa-miR-210, hsa-miR-221, hsa-miR-451, and hsa-miR-93) are shared between Li’s 69-miRNA signature and our 30-miRNA signature. Of the 11 miRNAs, 10 were upregulated and only one (miR-451) was downregulated in hepatocellular carcinoma in the 2 studies. This result provides an independent validation of our result. With regard to the correct classification rate, our 30-miRNA signature was 99.2% accuracy for distinguishing hepatocellular carcinoma from noncancerous liver in training set, which was verified in the test and independent sets. The accuracy of our signature was higher than all previously reported miRNA signatures.

In addition, there are 11 unique miRNAs (miR-296-1, miR-486-5p, miR-516a-5p, miR-612, miR-625*, miR-634, miR-675, miR-766, miR-933, and miR-940) in our 30-miRNA signature, which have not been reported in hepatocellular carcinoma by others because many of them were only recently identified and made available in the public database and the number of miRNAs included in our study are much more than those in previous studies. The studies on miRNA differential expression between hepatocellular carcinoma and noncancerous liver suggested that miRNA expression profile might be a potential diagnostic tool in hepatocellular carcinoma clinical practice and provides a clue for exploring molecular mechanism of hepatocarcinogenesis and discovering new molecular targets for hepatocellular carcinoma therapy.

With the custom microarray, we also identify a 20-miRNA signature that is significantly associated with OS and DFS in 60 patients of the training set. Each of the 20 miRNAs was also significantly associated with hepatocellular carcinoma survival by Cox model. This 20-miRNA signature was validated in 50 patients of the test set and 56 patients of the independent set. These results indicate that the signature is robust and reliable. The survival-related 20-miRNA signature should provide a valuable approach to help clinicians make better informed decision on patient with hepatocellular carcinoma management.

Compared with a survival-related 19-miRNA signature detected in 25 U.S. patients with hepatocellular carcinoma by qRT-PCR with 196 miRNAs reported by Liang and colleagues in 2008 (28), our 20-miRNA signature shared no common miRNA with the 19-signature. A potential reason is that the hepatocarcinogenesis process of U.S. patient is very different from that of Chinese patient, which may reflect the fact that majority of US patients with hepatocellular carcinoma carry HCV, whereas Chinese patients are mainly infected with HBV. Another possible explanation for the distinct discrepancy is that the number of miRNAs in our study was nearly 4 times more than that in the study conducted by Liang and colleagues. In yet another hepatocellular carcinoma miRNA profiling study using a microarray containing about 225 human miRNA probes, Budhu and colleagues identified a metastasis-related 20-miRNA signature (30). In comparison with the miRNAs in our survival 20-miRNA signature, only one miRNA (hsa-miR-15a) is common even though the metastasis-related signature was also reported to be associated with outcome of hepatocellular carcinoma. Six miRNAs of our 20-miRNA signature were identified recently and deposited into the miRBase database and not included in microarray conducted by Budhu and colleagues. Thus, our 20-miRNA signature seems to be more representative and comprehensive than other signatures.

In univariate Cox analysis, our 20-miRNA signature was a significant predictor in the training, test, and independent sets (Supplementary Table S4), respectively. In multivariate Cox analysis, the 20-miRNA signature was a significant independent predictor in training set and a marginally significant independent predictor in test set, but not an independent predictor in independent cohort (Supplementary Table S4). However, when combined with the test set and independent cohort, the signature was a significant independent predictor with multivariate Cox analysis (Supplementary Table S5). These results suggest that this signature should be also an independent predictor in the test set or independent cohort if the sample size is increased.

In the present study, HBeAg was shown to be an independent predictor for survival of patients with hepatocellular carcinoma, which is not often reported in the published literature. HBV infection is a major cause of hepatocellular carcinoma in some areas, especially in China, and HBeAg is a marker of active HBV replication and infection, which aggravates the impaired liver function of patients with hepatocellular carcinoma. Liver function status is an important predictor of hepatocellular carcinoma survival and one of the main factors of the hepatocellular carcinoma clinical staging system. Hence, it is possible that HBeAg is associated with hepatocellular carcinoma survival. Some studies on patients with hepatocellular carcinoma with HBV infection indicate that HBeAg is an independent prognostic factor or correlated with hepatocellular carcinoma survival (31, 32). Furthermore, a lot of studies indicate that HBV infection is a predictive factor for hepatocellular carcinoma survival (33, 34). Therefore, our result provides further support that HBeAg was a predictor for hepatocellular carcinoma survival.

In conclusion, we used a large comprehensive set of miRNAs to identify a new 30-miRNA signature that can discriminate hepatocellular carcinoma tissues from noncancerous liver tissues and a novel 20-miRNA signature that can predict the survival of patients with hepatocellular...
carcinoma in the training set. Both signatures are validated in the test and independent sets. Most miRNA species of our signatures have not been reported previously in hepatocellular carcinoma. The findings in the present study have the potential to provide novel molecular approaches for diagnosis and prognosis of patients with hepatocellular carcinoma. The miRNAs of the 2 signatures may also be important for studying pathogenesis of hepatocellular carcinoma and identifying potential targets for hepatocellular carcinoma therapy. Furthermore, the study on these miRNAs will provide new insights into the development and progression of hepatocellular carcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: R. Wei, M. Shi, Y.-F. Yuan, H.-Y. Wang
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Writing, review, and/or revision of the manuscript: R. Wei, G.-L. Huang, M. Shi, X.F.S. Zheng, H.-Y. Wang

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