Combined low miRNA-29s is an independent risk factor in predicting prognosis of patients with hepatocellular carcinoma after hepatectomy
A Chinese population-based study

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Abstract
The prediction of prognosis of hepatocellular carcinoma (HCC) following partial hepatectomy is still an unresolved issue. The aim of this study is to identify the association between miRNA-29s family and the prognosis of patients with HCC in a large Asian cohort. We retrospectively reviewed 122 patients with HCC managed in our institution between 2008 and 2015. The expression of miRNA-29s was detected by real-time polymerase chain reaction (PCR). Prognostic factors were evaluated using Kaplan–Meier curves and Cox proportional hazards models. For the entire cohort of 122 patients, the normalized real-time PCR results showed that miRNA-29s (miR-29a, miR-29b, and miR-29c) were deregulated in tumor tissues as compared with corresponding nontumorous tissue samples. We then performed survival analysis to investigate the prognostic value of miRNA-29s. We found that low miR-29b was associated with a decreasing 5-year overall survival (OS) rate from 70.2% to 39.1% and low miR-29c was associated with a decreasing 5-year OS rate from 53.6% to 23.7%. We further conducted multivariate Cox proportional hazards analysis adding the variable of combined low miR-29b and low miR-29c. The results demonstrated that combined low miR-29b and miR-29c was an independent prognostic factor of patients with HCC. In conclusion, we found that the miRNA-29s were down-regulated in tumor tissues as compared with corresponding nontumorous tissue samples. Combined low miR-29b and miR-29c was an independent prognostic factor of patients with HCC.

Abbreviations: AFP = alpha-fetoprotein, CT = computed tomography, HCC = hepatocellular carcinoma, HR = hazard ratio, miRNAs = microRNAs, MRI = magnetic resonance imaging, OS = overall survival, PCR = polymerase chain reaction, ROC = receiver-operating characteristic, SD = standard deviation, TNM = tumor-node-metastasis.

Keywords: hepatocellular carcinoma, miRNA-29s, prognosis

1. Introduction
Hepatocellular carcinoma (HCC) is the 5th most frequently diagnosed cancer worldwide and the 3rd most frequent cause of cancer death, with the highest incidence in Asian and especially in China. Partial hepatectomy remains the most commonly used curative therapy modality for HCC. Although the prognosis of patients with HCC has been improved recently, the survival outcomes of patients with HCC following surgical resection may vary, as several factors are associated with the prognosis of HCC, including completeness of tumor removal, serum alpha-fetoprotein (AFP) levels, tumor size, tumor multi-focality, microvascular or macrovascular invasion, distant metastases, etc. The poor prognosis of patients with HCC is attributed to the lack of effective means of prognosis prediction. Few patients underwent tumor recurrence monitoring based on the prognostic factors. Discovery of an effective and reliable modality would play a pivotal role in improving the prognosis of patients with HCC.

MicroRNAs (miRNAs) are emerging as a new class of regulatory molecules involved in numerous biologic processes. MiRNAs are a large class of small, noncoding, single-stranded RNAs, 19 to 23 nucleotides in length, and constitute a novel class of gene regulators. By targeting multiple transcripts, miRNAs are involved in biological processes including cell differentiation, proliferation, apoptosis, and metastasis. miRNA deregulation is a common feature of human malignancies as they control the expression of oncoproteins or tumor suppressors acting as oncomiRNAs or tumor suppressor miRNAs themselves.

The human miRNA-29 family, which has 3 main members including miR-29a, miR-29b, and miR-29c, plays crucial role in variety of pathophysiological processes. This group of miRNAs has been extensively studied and has been shown to be down-regulated in several cancers, such as gastric, peripheral nerve sheath tumors, esophageal squamous cell carcinoma, melanoma, breast cancer, and others. Fabbri et al. demonstrated that expression of miR-29a, miR-29b, and miR-29c is down-regulated in nonsmall cell lung cancer. Plaisier et al. found that the miR-29 family inhibited specific genes associated with invasion and metastasis of lung adenocarcinoma. In a variety of
malignant tumors, the decreased expression of miR-29c led to the dysregulation of its downstream target genes that were involved in epigenetic modification, metastasis, and cell proliferation.

With respect to patients with HCC, there were few studies focusing on miRNA-29s roles in the pathogenesis and prognosis of patients with HCC after hepatic resection. Based on the crucial roles of miRNA-29s family in various cancers, we detected the expression of miRNA-29s in patients with HCC and identified the association between miRNA-29s family and the prognosis of patients with HCC in a large Asian cohort of a single institution. We also aimed to explore the interaction of miRNA-29s with other independently risk factors in the process of affecting prognosis of HCC.

2. Materials and methods

2.1. Patients and tissue samples

A total of 122 patients with primary HCC who underwent a curative liver resection were included in this retrospective study. These patients were diagnosed as HCC in Department of Hepatobiliary and Laparoscopic Surgery, Renmin Hospital of Wuhan University. The tissues were immediately frozen in liquid nitrogen after surgical removal and stored at −80°C until use. HCC diagnosis was based on World Health Organization criteria. Tumor staging was determined according to the 6th edition of the tumor-node-metastasis (TNM) classification of the International Union against cancer. The characteristics of patients were shown in Table 1. The study was approved by the Research Ethics Committee and Informed consent was obtained from all patients.

2.2. Diagnosis and treatment

After a detailed history and a thorough physical examination, blood was collected for liver function test and tumor markers. Contrast-enhanced computed tomography (CT) and/or magnetic resonance imaging (MRI) was used to confirm the TNM staging at diagnosis.

Liver resection was carried out, taking note of the tumor diameter, location, tumor extension, and estimated volume of the remaining liver. Liver resection was performed following Couinaud segments, sectors, and hemilivers.

2.3. RNA extraction and miRNA quantification

Total RNA was extracted from fresh frozen tissues using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol; 100 to 150 mg tumor tissue and nontumor tissue were used to extract RNA. We typically extracted 2 to 9 μg of total RNA, and OD260/280 ratios typically ranged from 1.8 to 2.0, indicating high RNA purity; 10 ng of total RNA was used for each miRNA quantification. miRNA detection was performed run on the Eppendorf Mastercycler EP Gradient S (Eppendorf, Germany) using commercial assays (TaqMan microRNA assays; Applied Biosystems, Foster City, CA) for miRNAs. Relative quantification was calculated using 2^−ΔΔCt, where Ct is cycle threshold. Normalization was performed with universal small nuclear RNA U6 (RNU6B). Each sample was examined in triplicate, and the mean values were calculated. mRNA levels in tumor samples/nontumor samples of 0.5-fold were defined as under-expression of the gene, whereas a ratio of 2.0-fold was defined as over-expression.

2.4. Follow-up

Postoperative serum AFP and abdominal ultrasound were carried out in all patients monthly. Patients received abdominal contrast-enhanced CT scan or MRI once every 3 months in the first 2 years after surgery, and once every 6 months thereafter. Further investigations were carried out when clinically indicated or when tumor recurrence was suspected. Outcome definitions: Complete
Resection was defined as resection of all tumor sites on the basis of surgical findings and postsurgical images. Overall survival (OS) was defined as the period from the date of surgery until death or last contact. Patients who did not experience an event were censored on the date of last contact. OS was defined as the period from the date of surgery until an occurrence of death or last contact.

### 2.5. Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) and compared using a 2-tailed unpaired Student t test; categorical variables were compared using χ² or Fisher analysis. The cut-off of AFP level was defined by the receiver-operating characteristic curve analysis.[14] Life-table estimates of survival time were calculated according to the Kaplan–Meier methodology.[15] The Greenwood formula was used for the SD. A Cox proportional hazards regression approach[16] was chosen for the evaluation of OS as the primary end-point. Potential prognostic variables were analyzed both univariately with 1 factor taken at a time, and then in a multivariate model combining all factors. Results were showed as hazard ratios (HRs) and their 95% confidence intervals. An HR > 1 indicated an elevated risk with respect to the reference category. A confidence interval which did not include the value 1 indicated statistical significance at the 5% level. It should be noted that this was a retrospective evaluation and therefore statistical significance should be interpreted with caution. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 15.0, SPSS Inc., Chicago, IL). A value of P < .05 was considered to be statistically significant in all the analyses.

### 3. Results

#### 3.1. Patients’ characteristics

One hundred twenty-two patients with HCC after hepatectomy were recruited into this study. The median follow-up was 6.5 years (ranging from 6.2 months to 8.2 years). The baseline characteristics of patients at diagnosis were summarized in Table 1. Overall, most patients were male (M:F = 2.49:1). Most patients had tumor size < 5 cm (63.1%), microvascular invasion (62.3%), and solitary tumor (81.1%). The under-expression of miR-29a, miR-29b, and miR-29c was 42.6%, 78.7%, and 35.3%, respectively.

#### Table 2

| Variables | 1-y OS, % | 3-y OS, % | 5-y OS, % |
|-----------|-----------|-----------|-----------|
| Overall   | 87.5      | 53.3      | 44.1      |
| AFP, ng/mL| <400      | 90.3      | 63.4      | 53.4      |
|           | >400      | 84.5      | 44.4      | 34.3      |
| Median diameter, cm| | | | |
| <5        | 96.1      | 63.1      | 53.1      |
| >5        | 72.4      | 36.3      | 28.6      |
| Microvascular invasion| | | | |
| Yes       | 95.5      | 65.6      | 57.1      |
| No        | 80.1      | 45.9      | 34.4      |
| Tumor number| | | | |
| Solitary  | 88.7      | 56.5      | 48.7      |
| Multiple  | 78.1      | 39.7      | 24.8      |
| MRNA-29a  | | | | |
| High      | 78.6      | 52.6      | 46.9      |
| Low       | 68.4      | 50.4      | 41.6      |
| MRNA-29b  | | | | |
| High      | 94.7      | 76.8      | 70.2      |
| Low       | 83.4      | 54.2      | 39.1      |
| MRNA-29c  | | | | |
| High      | 96.5      | 64.7      | 53.6      |
| Low       | 81.2      | 43.2      | 23.7      |

AFP = alpha-fetoprotein, OS = overall survival.

Figure 1. miR-29a (A, B), miR-29b (C, D), and miR-29c (E, F) expressions in tumor and nontumorous tissues detecting by relative reverse transcription polymerase chain reaction quantitation.
3.2. The expression of miRNA-29 family members was significantly down-regulated in human HCC tissues

We detected miRNA-29s levels in 30 paired tumor and nontumor tissues of patients with HCC after hepatectomy. The normalized real-time PCR results showed that all 3 miRNAs (miR-29a, miR-29b, and miR-29c) were deregulated in tumor tissues as compared with corresponding nontumorous tissue samples ($P < .01$, showed in Fig. 1).

3.3. Survival descriptions with different risk factors

For all 122 patients, the overall 1-, 3-, and 5-year OS were 87.5%, 53.3%, and 44.1%, respectively (Table 2). Descriptive survival statistics and Kaplan–Meier curves suggested that AFP levels $> 400$ ng/mL, tumor size $> 5$ cm, microvascular invasion, tumor multifocality, and low expression of miR-29b and miR-29c had prognostic significance in this relatively selected cohort (Fig. 2). Low expression of miR-29a showed no significantly prognostic value (not shown). AFP $> 400$ ng/mL was associated with a decreasing 5-year OS rate from 53.4% to 34.3% ($P = .010$, Fig. 2A). Tumor size $> 5$ cm was associated with a decreasing 5-year OS rate from 53.1% to 28.6% ($P < .001$, Fig. 2B). Microvascular invasion was associated with a decreasing 5-year OS rate from 57.1% to 34.4% ($P = .009$, Fig. 2C). Multiple tumors was associated with a decreasing 5-year OS rate from 48.7% to 24.8% ($P = .036$, Fig. 2D). Low miR-29b was associated with a decreasing 5-year OS rate from 70.2% to 39.1% ($P = .020$, Fig. 2E). Low miR-29c was associated with a decreasing 5-year OS rate from 53.6% to 23.7% ($P = .012$, Fig. 2F). After that we performed further analysis to combined low miR-29b and low miR-29c as 1 predictor. Survival analysis showed that combined low miR-29b and low miR-29c was associated with a decreasing 5-year OS rate from 51.7% to 30.5% ($P = .006$, Fig. 3).

Figure 2. Overall survival estimates. (A) The overall survival curves stratified by AFP levels ($P = .010$); (B) the overall survival curves stratified by tumor size ($P < .001$); (C) the overall survival curves stratified by MVI ($P = .009$); (D) the overall survival curves stratified by tumor number ($P = .036$); (E) the overall survival curves stratified by miR-29b levels ($P = .020$); and (F) the overall survival curves stratified by miR-29c levels ($P = .012$). AFP = alpha-fetoprotein, MVI = microvascular invasion.
3.4. Cox proportional hazard analysis

Cox proportional hazard models were then used to quantify the prognostic significance of risk factors after multivariable adjustment. A multivariable analysis was performed to assess the factors that demonstrated significant effects as in univariate analysis. We identified that HR levels >400 ng/mL, tumor size >5 cm, microvascular invasion, tumor multifocality, and low expression of miR-29b and miR-29c were associated with a worse prognosis in the univariable analysis. After adjusting for competing risk factors, we found that low expression of miR-29b (HR: 1.269, P=.067) and miR-29c (HR: 1.179, P=.102) showed no significantly prognostic value in the multivariable adjusted analysis (Table 3). We believed that some interaction or collinearity existed among these 2 variables or with other significant factors. We further conducted multivariate Cox proportional hazard analysis adding the variable of combined low miR-29b and low miR-29c. The results demonstrated that combined low miR-29b and miR-29c (HR: 2.561, P=.12) was an independent prognostic factor of patients with HCC (Table 3).

4. Discussion

Although the prognosis of patients with HCC had been improved since multiple therapeutic modalities had been applied, the OS was still unsatisfactory. Many risk factors were associated with the prognosis of patients with HCC after hepatectomy. Based on Asian population, researchers had confirmed prognostic factors including AFP levels, vascular invasion, tumor metastases, multiple tumors, and other clinicopathological features. Besides with these factors, many novel biomarkers had been investigated and explored. However, the prediction of prognosis of patients with HCC following partial hepatectomy is still an unresolved issue.

MiRNAs could bind to complementary sites on the 3’ untranslated region of target genes, and consequently regulate post-transcriptional gene expression by mRNA degradation and translational repression. The role of miRNAs in cancer has often been discussed. There is increasing evidence that miRNAs widely participate in the regulation of oncogenes or tumor suppressors in cancer. A growing amount of evidence has suggested that miRNAs play important roles as prognostic and predictive biomarkers in cancers. Recent studies demonstrated that the miR-29 family is involved in many diseases and pathophysiological processes. For example, miR-29 was found to play an important role in aneurysm formation, systemic sclerosis, preeclampsia, and irritable bowel syndrome, and also was found to have an impact on pathophysiological processes of liver fibrosis, cardiac fibrosis, renal fibrosis, and myocardial ischemia–reperfusion injury. More and more research indicates that miR-29 participates in the processes of apoptosis, proliferation, and epithelial-mesenchymal transition in cancer.

In the present study, our results showed that miRNA-29s were under-regulated in tumor tissues as compared with corresponding nontumorous tissue samples. Moreover, we found that low miR-29b was associated with a decreasing 5-year OS rate from 70.2% to 39.1% and low miR-29c was associated with a decreasing 5-year OS rate from 53.6% to 23.7%, respectively. We further conducted multivariate Cox proportional hazard analysis after combined low miR-29b and low miR-29c as 1 variable. The result demonstrated that combined low miR-29b and miR-29c was an independently prognostic factor of patients with HCC.

To the best of our knowledge, there are many recognized prognostic and predictive markers for HCC, including several clinicopathological features and serum biomarkers. The present study is the first to explore the potential implications for miRNA-29s related to HCC prognosis. Meanwhile, there are limitations of this study: the study was based on the data of Chinese patients with a majority background of HBV infection. There were many background factors influencing this disease including alcohol consuming, virus hepatitis, obesity, and type 2 diabetes mellitus. Further studies were needed to take these factors into consideration; in this study, there were 70 (57.4%) patients with cirrhosis which progressed into HCC.
expression of miRNA-29s influenced by cirrhosis, stratified studies were needed though we performed multivariate analysis; and this is a retrospective study and the sample size is small, further studies with larger samples or multicenters are needed. In conclusion, this study showed that the miRNA-29s were down-regulated in tumor tissues as compared with corresponding nontumorous tissue samples. Combined low miR-29b and miR-29c was an independent prognostic factor of patients with HCC.

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