Over-Expressions of Serum miR-182-5p, miR-363-3p, and miR-378a-3p serve as Biomarkers in Hepatocellular Carcinoma

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Abstract
Serum α-fetoprotein (AFP) has been used as an early diagnostic biomarker of hepatocellular carcinoma (HCC), however the sensitivity and specificity of detection are poor. Therefore, new markers remain to be explored. MicroRNAs (miRNAs) are a class of small RNAs which could contribute to tumorigenesis by interaction with targeted mRNAs. miRNAs are abundant in the circulating blood and are potentially useful for early diagnosis of hepatocellular carcinoma. In this study, we screened a group of candidate miRNAs (miR-182-5p, miR-363-3p, and miR-378a-3p) in serum, which were found to be up-regulated in HCC patients compared with in the healthy controls. Analysis of the receiver operating characteristic (ROC) curve suggested that these three serum miRNAs had merits in the diagnosis of HCC (AUC=0.802, 0.845, 0.880, respectively). Furthermore, these candidate miRNAs can also differentiate HCC patients with negative AFP level from the healthy controls (AUC=0.865, 0.930, 0.970, respectively). In conclusion, our results suggested that serum miR-182-5p, miR-363-3p, and miR-378a-3p might be applied as potential non-invasive biomarkers for HCC diagnosis, which is especially important for AFP-negative patients.

Keywords: MicroRNA; Hepatocellular carcinoma; AFP-Negative biomarker

Introduction
Hepatocellular carcinoma (HCC) is one of the most common cancers and represents a leading cause of malignancy-related death, with approximately 750,000 new cases and about 700,000 deaths annually worldwide [1]. Due to the lack of effective early detection or therapeutic strategy, the majority of HCC patients are diagnosed when they reach medium or even terminal stage, which leads to high mortality [2]. At present, surgical resection or liver transplantation are the main therapeutic methods to increase survival for HCC patients. The overall 5-year survival rate for liver cancer is about 14% worldwide [1]. In clinical diagnosis for primary HCC, α-fetoprotein level is a main indicator. However, its sensitivity and specificity have been far from satisfactory [3]. Approximately 20% of HCC patients with low AFP level are poorly diagnosed [4]. Therefore, screening potential biomarkers to effectively detect early tumor development is an important step towards HCC diagnosis and mortality reduction [5]. MicroRNAs are a class of endogenous, noncoding RNAs with ~22 nucleotides in length that are cleaved from 70- to 100-nt hairpin-shaped precursors [6]. miRNAs regulate gene expression at the post-transcriptional level by binding partial sequence to the 3’ untranslated region (3’UTR) of target mRNAs. The binding interaction causes translational inhibition and/or mRNA degradation [7]. Moreover, dysregulation of miRNAs expression may contribute to tumorigenesis by inhibiting the expression of tumor suppressor genes or promoting the expression of proto-oncogenes [8]. Several studies have demonstrated that miRNAs have high stability in human serum or plasma due to protection from RNases [9-11], suggesting their potential use as diagnostic biomarkers. Therefore, profiling miRNA expression pattern may help to clarify the biological functions of miRNAs in hepatocarcinogenesis [6], which has considerable clinical value.

Materials and Method
Patient characteristics and clinical features
This study was approved by the Institutional Review Board of Zhejiang Sci-Tech University and Hangzhou Xixi Hospital (Hangzhou, China). Written informed consent was obtained from each participant. Furthermore, this study was conducted in accordance with ethical statement in the Declaration of Helsinki by the World Medical Association. A total of 77 participants including 52 HCC patients and 25 normal subjects were recruited into this study. The clinical pathological characteristics of the participants are summarized in Table 1.

Solexa sequencing and validation
For the initial analysis of miRNA profile in HCC, the study was divided into two phases. The first phase of this study was designed to screen differential expression of miRNAs between HCC patients and healthy controls. Sample pooling was considered as a powerful, cost-effective, and rapid means to identify the changes in a gene expression source are credited.

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profile [12]. For Solexa sequencing, 5 HCC patients serum were pooled together and 5 healthy control samples were pooled together, respectively, to reduce the individual difference [13]. Small RNA molecules with the length of less than 50 bases were ligated with a pair of Solexa adaptors, reverse transcribed and amplified. The cDNA library was directly sequenced using Illumina's Solexa HiSeq 2000 Sequencer (Illumina, San Diego, USA). Finally, clean reads were compared with the miRBase database (version 20.0), and the abundance value of each known miRNA was normalized using ''transcripts per million (TPM)'' in both samples [14]. The differential expression of miRNA between two groups was revealed by clustering analysis. To validate this result, all samples (52 HCC and 25 controls) were further studied for the expression levels of interested miRNAs by real-time quantitative polymerase chain reaction (qRT-PCR) assay. Serum RNA (2.5 μl) from each sample was used in reverse transcription reaction, using 2 mM miRNA-specific stem-loop primers and M-MLV reverse transcriptase according to the instruction of the manufacturer (Takara, Dalian, China). Primers for miR-182-5p, miR-363-3p, miR-378a-3p and RNU6 were designed by Primer premier 5 software (Premier Inc., Ontario Ottawa, Canada) and the sequences were listed in Supplementary Table S1. qRT-PCR was performed with ABI Prism 7500 RT-PCR system (Applied Biosystems, Foster City, USA), using Takara SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara). Each amplification reaction was conducted in a total volume of 20 μl, containing 1 μl cDNA (~10 ng/ reaction), 10 μl 2X SYBR Green PCR Master mix, 0.4 μl 50X ROX, 0.8 μl 10 mM primer mix, and 7.8 μl RNase-free water. Every sample was amplified in triplicates. The expression of the miRNAs in the HCC cases and the healthy controls was normalized relative to the expression of RNU6 which served as an internal reference gene [15-18]. The relative expression levels of selected miRNAs were then determined as ratios by dividing the mean of 2ΔCT values of cancer group from the mean of 2ΔCT values of the healthy controls [19,20].

**Statistical analysis**

Statistical analysis was performed using the Statistical Program for Social Sciences (SPSS) software (version 17.0, SPSS Inc., Chicago, USA). All data were presented as mean ± standard deviation (SD) [21]. The statistical differences of miRNA expression levels between experimental group and control group were analyzed by independent samples t test. P<0.05 was considered as statistically significant, P<0.01 was considered highly significant. The receiver operating characteristic (ROC) curve was available to evaluate the feasibility of serum miRNA as a biomarker for HCC early diagnosis [22,23]. A stepwise logistic regression model was used to select diagnostic microRNA markers based on the validating dataset. ROC curve and logistic regression

### Table 1: Summary of clinical details of subjects.

| Parameters                          | Sequencing set Healthy Control (n=5) | HCC patients (n=5) | Validation set Healthy Control (n=25) | HCC patients (n=52) |
|-------------------------------------|--------------------------------------|--------------------|---------------------------------------|---------------------|
| Age, median, years                  |                                      | 33                 | 51                                   | 29                  |
| Men, n(%)                           |                                      | 3, 60.0%           | 4, 80.0%                             | 19, 76.0%           |
| AFP (ng/ml)                         | ≤ 20                                  | 5                  | 1                                    | 25                  |
|                                     | >20                                   | 4                  |                                       | 26                  |
| Hepatitis B virus DNA copies (IU/ml)| ≤ 1000                                | 5                  | 2                                    | 25                  |
|                                     | >1000                                 | 3                  |                                       | 20                  |
| HBsAg level                         | negative                              | 5                  | 0                                    | 25                  |
|                                     | positive                              | 5                  |                                       | 2                   |
| Alanine aminotransferase (UI)       | ≤ 40                                  | 5                  | 1                                    | 25                  |
|                                     | >40                                   | 4                  |                                       | 29                  |
| Aspartate aminotransferase (UI)     | ≤ 40                                  | 5                  | 1                                    | 25                  |
|                                     | >40                                   | 4                  |                                       | 26                  |
| Intrahepatic metastasis             |                                      |                    |                                       |                     |
| Distant metastasis                  |                                      |                    |                                       |                     |
| Portal vein tumor thrombus          |                                      |                    |                                       |                     |
| Tumor size                          | ≤ 5                                   |                    |                                       |                     |
|                                     | >5                                    |                    |                                       |                     |
| TNM phase                           | I phase                               |                    |                                       |                     |
|                                     | II phase                              |                    |                                       |                     |
|                                     | III phase                             |                    |                                       |                     |
|                                     | IV phase                              |                    |                                       |                     |
analysis were performed with MedCalc (version 10.4.7.0.; MedCalc, Mariakerke, Belgium) software.

Results

RNA-Seq and microRNA screening

To obtain an expression profile of specific serum miRNAs for HCC, we employed the RNA-seq to compare the miRNA expression levels in HCC serum with that in the healthy controls. As shown in Supplementary Table S2, there was significant variation of serum miRNAs between different samples. From the Solexa sequencing results, 531 miRNAs were detected in 5 mixed HCC samples, while 799 miRNAs were detected in 5 mixed healthy samples, and 417 miRNAs were detected in both samples. It is important that the miRNAs called expressed are really expressed, then a conservative criterion of TPM ≥ 2 or ≥ 4 could be used [24]. In this study, we got rid the poorly expressed miRNAs with TPM<2. Among these shared-miRNAs, there were 88 miRNAs were up-regulated and 86 miRNAs were down-regulated in HCC samples, the proportion of miRNAs with fold change larger than 2 is around 41.7 %. MiRNAs with 2-folds higher expression in HCC cases than in healthy subjects were selected to search published literatures concerning these genes. MiR-182-5p was reported to be a good prognostic biomarker for glioma progression and patient survival [25]. MiR-363-3p acts as an oncogene in head and neck squamous cell carcinoma [26]. MiR-378a-3p was demonstrated to be an important molecule in various types of human cancer. In the design phase, we referenced the sequencing data of the paper titled "Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma" [27], and screened the abundantly expressed microRNAs in liver cancer tissue based on raw data of supplementary information they provided (Supplementary Table S3). MiR-182-5p, miR-363-3p, and miR-378a-3p were over-expressed significantly in tumor tissue than in adjacent tissue, too. The study indicated the candidate miRNAs (miR-182-5p, miR-363-3p, and miR-378a-3p) may have potential significant in clinical diagnosis. The differential expression of miRNA between the control and HCC group was revealed by clustering analysis (Supplementary Figure S1).

Validation of RNU6 as an internal reference for quantification

In order to verify whether RNU6 could serve as an internal reference for quantification of serum miRNAs, we assayed the levels of several reference gene including GADPH, β-actin and RNU6 by qRT-PCR in 20 serum samples (10 healthy individuals and 10 HCC patients), and the CT values of these reference genes were used to calculate average expression stability value (M) with geNorm software. Gene with the lowest M value has the most stable expression in the test samples [28]. Data analysis with geNorm software showed that RNU6 had the smallest M value of 0.146 compared with GADPH (0.157) and β-actin (0.165), which indicated this small noncoding RNA is stably expressed in serum and is an optimal reference gene for the PCR quantification of other serum miRNAs [29].

Detection of candidate miRNAs profile in HCC samples versus healthy controls

In order to corroborate the RNA-seq results, we examined the expression levels of the target miRNAs in 52 HCC cases and 25 healthy controls by qRT-PCR. The profiles of miR-182-5p, miR-363-3p, and miR-378a-3p in serum samples were analyzed. CT values of miRNAs were normalized with the reference gene RNU6. Similar results were obtained in the qRT-PCR. The selected miRNAs (miR-182-5p, miR-363-3p, and miR-378a-3p) showed significantly higher expression in HCC patients than in the healthy controls (fold changes were 3.98, 6.78, and 9.03, respectively) (Figure 1, Supplementary Table S4). The differences were statistically significant (P<0.01). However, we also noticed that although HCC patients had statistically higher 10 average expression of the candidate miRNAs than the healthy controls, a certain degree of overlap appeared between these two groups. This was caused most probably by inter individual variation [22]. ROC curve analysis suggested that serum miR-182-5p, miR-363-3p, and miR-378a-3p levels were useful biomarkers for discriminating patients with HCC from the healthy controls. Area under the curve (AUC) was used as an accuracy index for evaluating the diagnostic performance of the selected miRNA panel. AUC of ROC curve analysis at 0.7-0.8 was considered inaccurate, AUC at 0.8-0.9 was considered reasonably accurate while AUC>0.9 was considered highly accurate [23]. The ROC curve of circulating miRNAs had an AUC of 0.802, 0.845, and 0.880, respectively (Figure 2). Among these results, we partly confirmed elevated miRNAs had high accuracy to distinguish HCC from the healthy controls. Worth special noting was that these three miRNAs can also differentiate HCC patients with negative AFP level from the healthy. As a HCC biomarker, serum AFP is currently used in clinical examination. In general, AFP<20 ng/ml is considered as normal and AFP>400 ng/ml as truly positive of cancer [30]. Compared with the healthy group, the miRNAs expression profile was significantly increased in HCC patients with low AFP (Supplementary Table S5). The qRT-PCR analysis demonstrated that the expression of miR-182-5p was increased approximately 3.01-fold in HCC patients (n=16) with AFP<20 ng/ml than in normal subjects (n=25, P=0.001). A similar trend was observed in expression profile of miR-363-3p, which was elevated 10.19-fold in AFP-negative patients (P=0.002). Furthermore, miR-378a-3p was over-expressed 10.68-fold
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in AFP-negative group ($P=0.001$) (Figure 3). ROC curve analysis of miR-182-5p, miR-363-3p, and miR-378a-3p for discriminating HCC patients with negative AFP level from the healthy controls yielded an AUC of 0.865, 0.930, and 0.970, respectively (Figure 4). The results illustrated that the tested miRNAs might be used as potentially specific molecular markers for the early diagnosis of HCC subgroup.
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Establishing the predictive microRNA panel

To estimate the risk of being diagnosed with HCC, the validating dataset (77 plasma samples) was used to establish a stepwise logistic regression model. All of the tested miRNAs (miR-182-5p, miR-363-3p, and miR-378a-3p) were shown to be significant predictors. The predicted probability of being diagnosed with HCC from the logistic regression model based on the three microRNA panel [13], logit (p=HCC) = -0.8959 + 0.06493 × miR-182-5p + 0.574 × miR-363-3p + 0.2861 × miR-378a-3p was used to construct the ROC curve. The diagnostic performance for the established microRNA panel was evaluated by using ROC analysis, and the AUC for the microRNA panel was 0.864.

Relationship of circulating miRNAs to pathology

It was reported that some unique miRNA signatures were associated with prognostic factors and disease progression in several cancers [2]. Therefore, we analyzed the correlation between circulating miRNAs expression and clinicopathological parameters. As shown in (Table 2), the correlation analysis showed that the serum miRNAs profile were directly associated with multiple factors, including age, AFP level, hepatitis B virus DNA copies and alanine aminotransferase (ALT), while there's no correlation with aspartate aminotransferase (AST) level. For example, miR-182-5p expression was negatively correlated with age ($P<0.05$), AFP level ($P=0.001$) and HBV DNA copies ($P=0.001$). MiR-363-3p profile was negatively correlated with age ($P=0.027$), AFP level ($P=0.048$) and positively correlated with ALT level ($P=0.023$).
The expression observed in miR-378a-3p, as similar as the former, was negatively correlated with AFP level ($P = 0.001$) and HBV DNA copies ($P = 0.001$), while positively correlated with ALT level ($P = 0.04$). Taken together, there are potential relationship between tested miRNAs and pathological factors. What's more, this result might help elucidate the mechanism of hepatocarcinogenesis.

**Discussion**

Accumulating evidence indicates that miRNAs play important roles in cancer initiation, propagation, and progression [31]. MiR-182 was localized in 7q31-34 and correlated with gene copy number reported to be abundantly expressed in human melanoma cell lines and tissue samples [32]. Recent reports showed that miR-182 was involved with the regulation of RGS17 expression by targeting its 3'UTR region in lung adenocarcinomas [33], while another study discovered that up-regulation of miR-182 resulted in the down-regulation of metastasis suppressor 1 and increased potential invasive of hepatocellular cell lines [34]. Besides, miR-182 was also considered as a significant prognostic biomarker for glioma progression and patients survival [25]. It suggested that miR-182 might serve as a biomarker for other types of cancer. Limited data was available for miR-363-3p expression in human cancers. Several studies demonstrated that miR 363 acted as an oncogene in head and neck squamous cell carcinoma by directly targeting podoplanin [26], and appeared to be important in HPV pathogenesis [35]. Over-expression of miR-363, on the other hand, reduced tumorigenicity and metastasis in cell invasion assay and liver metastasis in vivo [36]. MiR-363 on Xq26.2 was predicted to correlate with 672 mRNAs [37]. Inhibition of miR-363-3p induced hepatocellular tumorigenesis by promoting G1 to S phase progression, suggested that miR-363-3p might be a negative regulator of Myc and played yet unidentified roles in hepatocarcinogenesis [38]. For future studies about the regulatory mechanism of serum miRNAs in HCC, it will just be an interesting topic. Similarly, miR-378a-3p was not well studied, although a few reports suggested that it might exert different roles in different kinds of cancer pathogenesis. In human cancer, including colorectal cancer [39], non-small cell lung carcinoma [9] and gastric cancer [21], miR-378a was an important molecule. Various expression patterns were observed in culture and tumor tissue samples [40]. Thus more information about the miRNAs expression pattern and their roles in hepatocarcinogenesis is needed urgently. Above all these miRNAs, miR-363-3p and miR-378a-3p were known as passenger strand of the miRNA duplex and were generally regarded as being degraded in the miRNA biogenesis. Only the miRNA from the guide strand was used in transcription and protein synthesis [41]. Investigations indicated that well-conserved passenger strands, particularly conserved in seed sequences, may contribute to a network of cellular communication and molecular regulation [42]. Up to now, molecular mechanisms and roles of miRNA-3p species in the pathogenesis of cancer remain largely unknown. Possible miR-363-3p and miR-378a-3p binding sites by TargetScan/PicTar/RNA22/PITA/miR and software were listed in Supplementary Table S6. Our research provided help to understand and roles of miRNA-3p species in the pathogenesis of cancer remain unclear. Up to now, molecular mechanisms and roles of miRNA-3p species in the pathogenesis of cancer remain largely unknown. Possible miR-363-3p and miR-378a-3p binding sites by TargetScan/PicTar/RNA22/PITA/miR and software were listed in Supplementary Table S6. Our research provided help to understand and roles of miRNA-3p species in the pathogenesis of cancer remain unclear.

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