

# Circulating microRNA as a marker for predicting liver disease progression in patients with chronic hepatitis B

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## Abstract

**Introduction:** Hepatitis B virus (HBV) constitutes an important risk factor for cirrhosis and hepatocellular carcinoma (HCC). The link between circulating microRNAs and HBV has been previously reported, although not as a marker of liver disease progression in chronic hepatitis B (CHB). The aim of this study was to characterize miRNA expression profiles between CHB with and without cirrhosis or HCC. **Methods:** A total of 12 subjects were recruited in this study. We employed an Affymetrix Gene Chip miRNA 3.0 Array to provide universal miRNA coverage. We compared microRNA expression profiles between CHB with and without cirrhosis/HCC to discover possible prognostic markers associated with the progression of CHB. **Results:** Our results indicated 8 differently expressed microRNAs, of which miRNA-935, miRNA-342, miRNA-339, miRNA-4508, miRNA-3615, and miRNA-3200 were up-regulated, whereas miRNA-182 and miRNA-4485 were down-regulated in patients with CHB who progressed to cirrhosis/HCC as compared to those without progression. **Conclusions:** We demonstrated the differential expression of miRNA-935, miRNA-342, miRNA-339, miRNA-4508, miRNA-3615, miRNA-3200, miRNA-182, and miRNA-4485 between patients with HBV without cirrhosis/HCC and those who had progressed to these more severe conditions. These miRNAs may serve as novel and non-invasive prognostic markers for early detection of CHB-infected patients who are at risk of progression to cirrhosis and/or HCC.

**Keywords:** MicroRNA profiling. Chronic hepatitis B. Liver cirrhosis. Hepatocellular carcinoma.

## INTRODUCTION

Hepatitis B virus (HBV) infection represents a serious public health problem and constitutes a leading cause of liver cirrhosis and hepatocellular carcinoma (HCC)<sup>(1)</sup>. Approximately 240 million individuals are chronically infected with HBV worldwide with three quarters being within the Asia-Pacific region<sup>(1)</sup>. Among those with chronic HBV infection (CHB), up to 40% develop serious liver complications such as liver cirrhosis and HCC. Early identification of patients at risk of progression to cirrhosis and HCC will aid clinicians to initiate timely treatment strategies to prevent or at least reduce the risk of liver disease progression. Notably, the progression of HBV disease to liver cirrhosis and HCC is a multi-step process involving various genetic aberrations. In particular, emerging evidence has revealed the role of microRNAs (miRNAs) in various diseases including chronic inflammatory disease such

as ulcerative colitis and Crohn's disease<sup>(2)(3)</sup>, cancers such as chronic lymphocytic leukemia<sup>(4)(5)</sup>, and viral infection including hepatitis B infection<sup>(6)(7)</sup>.

MicroRNAs comprise small non-coding RNAs known to play vital roles in the post-transcriptional regulation of gene expression<sup>(8)</sup>. Additionally, miRNAs are involved in the regulation of several genetic processes such as apoptosis, cell differentiation, and development by preventing target mRNA translation<sup>(9)(10)</sup>. Furthermore, compared to mRNA, it has been demonstrated that the profiling of miRNAs enables the accurate classification of several tumors<sup>(11)</sup> to be involved in HBV positive cirrhosis and HCC<sup>(11)</sup>. In addition to their direct and indirect roles in the control of hepatitis B virus gene expression and replication, miRNAs are involved in immune response by controlling the cells responsible for innate immunity as well as the adaptive immune response through T-cell receptor signaling and antigen presentation (for example by modulating hematopoietic lineage differentiation)<sup>(12)(13)</sup>.

Several lines of evidence suggest that miRNAs control HBV replication<sup>(14)(15)(16)</sup>. Recent reports have suggested links between the aberrant expression of miRNAs in CHB and that in healthy controls<sup>(17)(18)(19)</sup>, as well as between HCC and non-tumor liver tissue<sup>(20)(21)(22)(23)</sup>. In addition, differential expression of some serum miRNAs, such as miRNA-122, miRNA-223,

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miRNA-375, miRNA-25, miRNA-21, let-7f, and miRNA-16 in patients with CHB as well as HBV-associated HCC have been reported to date<sup>(19) (24) (25)</sup>. In particular, a study by Xu *et al.*, which performed a comparison test between 48 CHB, 101 HBV-associated HCC, and 89 healthy subjects, indicated that serum miRNA-223, miRNA-122, and miRNA-21 were high in patients with CHB or HCC and that these miRNAs might serve as biomarkers for discriminating such patients from healthy controls<sup>(25)</sup>. Nevertheless, changes in circulating miRNA levels with respect to CHB progression to liver cirrhosis and HCC remains to be explored. Therefore, the aim of this study was to determine whether circulatory miRNA expression levels in patients with CHB could be used as a marker of liver disease progression in this group.

## METHODS

### Study design and patients

We recruited 12 subjects from the University of Malaya Medical Centre (UMMC) for this study. The expression profiles of microRNA in samples of participants in each group were detected using the Affymetrix miRNA array platform. The diagnosis of CHB was determined as positive for hepatitis B surface antigen (HBsAg) for more than a period of 6 months. Liver cirrhosis in patients with CHB was confirmed through histological evidence or a combination of clinical, biochemical, and radiological indication of cirrhosis<sup>(26)</sup>. Diagnosis of HCC was based on the radiological evidence of a liver mass with arterial hypervascularity and washout in the venous-delayed phase on dynamic imaging and/or by liver histology<sup>(27)</sup>. Healthy subjects were recruited from among blood donors at UMMC. The study procedure was carried out in accordance with the Helsinki Declaration and the study protocol (MEC Ref. No: 938.42) was approved by the medical ethics committee of the UMMC. A written consent form was signed by each individual.

### Sample collection

We collected 3mL blood into a Tempus™ Blood RNA Tube (Applied Biosystems, Foster City, CA, USA) containing 6 mL stabilizing reagent, from each participant. Immediately after the tempus tube was filled, the blood was stabilized by shaking the contents of the tube vigorously for 10 seconds to ensure that the stabilizing reagent made uniform contact with the sample.

The use of the Tempus blood ribonucleic acid (RNA) tubes provided a direct and convenient method to isolate high quality RNA from whole blood. After the blood was drawn into the Tempus tube and mixed with the reagent, cell lysis (whole blood cells) occurred immediately. Effectively, the stabilizing reagent selectively precipitates total RNA in a single step without any requirement for pre-treatment of blood prior to RNA purification.

### RNA extraction and quality control

Total RNA including microRNA was isolated from whole blood using the Preserved Blood microRNA Purification Kit I (Norgen Biotek Corporation, Thorold, ON, Canada) according to the manufacturer's protocol. This kit provided the rapid

isolation and purification of microRNA by removal of large RNAs and capture of microRNAs, which can be used in various downstream applications such as microarray analysis. The RNA purity and concentration was verified using the Nano-drop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of total RNA was determined by using an Agilent 2100 bio-analyzer (Agilent, Palo Alto, CA, USA) following the manufacturer's protocol. High quality RNAs with RNA Integrity Number (RIN)  $\geq 8$  and high purity with an optical density of 1.8-2.0 (260/280 ratio) were selected for further analysis.

### MicroRNA profiling

We tested 12 samples using Affymetrix Gene Chip® miRNA 3.0 Arrays (each containing 2999 probe sets). miRNA labeling was performed using the FlashTaq Biotin HSR RNA Labeling kit (Affymetrix), 10rxn (P/N 901910). For the labeling step, a minimum of 130ng total RNA was used for poly (A) tailing. Briefly, nuclease free water was used to adjust the volume of RNA to 8 $\mu$ L, to which, 2 $\mu$ L RNA Spike Control Oligos was added. The ATP mixture was diluted in 1mM Tris and the Poly (A) tailing master mix was prepared according to the Affymetrix protocol. After the addition of 5 $\mu$ L master mix to the 10 $\mu$ L RNA Spike Control Oligos, the mixture was incubated at 37°C for 15 min. Approximately 15 $\mu$ L of tailed RNA was utilized for the ligation step by adding 4 $\mu$ L 5X FlashTaq Biotin HSR ligation mixture followed by 2 $\mu$ L T4 deoxyribonucleic acid (DNA) ligase to each sample, then incubating at room temperature for 30 min. After the reaction was stopped through the addition of 2.5 $\mu$ L HSR stop solution, 23.5 $\mu$ L ligated sample was added. The Enzyme Linked Oligo Sorbent Assay quality control was performed prior to array hybridization according to the manufacturer's procedure. A volume of 21.5 $\mu$ L biotin labeled sample was then used for hybridization on Affymetrix Gene Chip® miRNA 3.0 Arrays. After preparing the oven, 110.5 $\mu$ L hybridization cocktail was added to the 21.5 $\mu$ L biotin-labeled sample according to the manufacturer protocol and incubated at 99°C for 5 min, then at 45°C for 5 min. A total of 130 $\mu$ L was finally inserted into the arrays, placed in the oven trays, then transferred to the hybridization oven for incubation at 48°C and 60rpm for 18h. To proceed to washing and staining, the hybridization cocktail from each array was removed, transferred to a new tube, and filled with array holding buffer. The washing and staining was then followed by placement of the arrays in fluidics station 450. The arrays were then washed and stained with Buffer A, Buffer B, and cocktail 1 and 2 followed by scanning using Affymetrix Command console software (version 1.3.1). After scanning, the raw data files produced at the end of the array scan (CEL files) were extracted for additional analysis using Transcriptome Analysis Console (TAC) software and Ingenuity Pathway Analysis (IPA).

### Quality control

The raw data obtained from Affymetrix were analyzed using Expression Console software (Affymetrix) for normalization and quality. Successful labeling and ligation were confirmed by the presence of background signal intensity higher than 1,000

compared to the control oligo 2, 23, 29, 31, and 36 RNA probe sets according to the manufacturer protocol.

### Identification of differentially expressed miRNAs

Expression analysis of microRNAs in the samples was carried out using Affymetrix TAC software. TAC enabled the identification of differentially expressed microRNAs in different groups. It provided the fold changes of microRNAs that were differentially expressed between chronic HBV with and without cirrhosis/HCC. An analysis of variance (ANOVA) p value of less than 0.05 was considered for determination of the most differentially expressed microRNAs between CHB with and without cirrhosis/HCC.

## RESULTS

To identify differentially expressed miRNAs between CHB with and without cirrhosis/HCC, miRNA microarray

was performed using 12 samples (n=4 for CHB without cirrhosis/HCC, n=4 for CHB with cirrhosis/HCC and n=4 for Healthy control) that were matched in terms of age, sex (only males were chosen), and ethnicity (only the Chinese ethnic group was chosen). The demographics of the study subjects are shown in **Table 1**. Microarray data revealed a number of differentially expressed microRNAs between CHB with and without progression to cirrhosis/HCC as shown in **Table 2**. We identified 8 detectable microRNAs with p value < 0.05 and fold expression change  $\geq 2$  or  $\leq -2$  between the CHB with cirrhosis/HCC and CHB without cirrhosis/HCC groups.

## DISCUSSION

It has been demonstrated that circulating miRNAs constitute ideal biomarkers in cancer as well as HBV associated liver disease owing to their stability in the circulation<sup>(8)(18)(19)</sup>. Notably, miRNAs have also been reported to play both oncogenic and

**TABLE 1**  
Demographics of the study subjects.

	HBV infected without cirrhosis/HCC (n = 4)	HBV infected with cirrhosis/HCC (n = 4)	Healthy controls (n = 4)
Age (years)	55	60	49
Median range	40–73	45–76	40–55
Ethnicity (Chinese)	4 (100.0%)	4 (100.0%)	4 (100.0%)
Gender (Male)	4 (100.0%)	4 (100.0%)	4 (100.0%)
HBV DNA	+	+	-
HBs Ag	+	+	-
HCV RNA	-	-	-

**HBV:** hepatitis B virus; **HCC:** hepatocellular carcinoma; **DNA:** deoxyribonucleic acid; **HBs Ag:** hepatitis B surface antigen; **HCV:** hepatitis C virus; **RNA:** ribonucleic acid.

**TABLE 2**  
Differentially expressed miRNAs between CHB with and without cirrhosis/HCC.

Transcript cluster ID	Fold change*	ANOVA p-value**
hp_hsa-mir-935_st	2.83	0.030138
hsa-miR-342_st	2.53	0.044610
hp_hsa-mir-339_st	2.27	0.039739
hp_hsa-mir-4508_st	2.27	0.041339
hsa-miR-3615_st	2.08	0.028634
hsa-miR-3200_st	2.01	0.001203
hsa-miR-182_st	-2.03	0.019457
hp_hsa-mir-4485_st	-2.29	0.040389

**miRNA:** microRNA; **CHB:** chronic hepatitis B; **HCC:** Hepatocellular carcinoma; **ID:** Identity; **ANOVA:** Analysis of variance. \*The results are shown as the mean fold change in miRNA expression of CHB with cirrhosis/HCC versus CHB without cirrhosis/HCC. Fold expression changes  $\geq 2$  or  $\leq -2$  were considered significant. \*\*p-value < 0.05 was considered significant.

tumor suppressor roles<sup>(28)</sup>. In the current study, the microarray results revealed 8 microRNAs that were differentially expressed between chronic HBV with progression to cirrhosis/HCC and chronic HBV without progression. miRNA-935, miRNA-342, miRNA-339, miRNA-4508, miRNA-3615, and miRNA-3200 exhibited  $\geq 2$ fold expression in the progression group as compared to the group without liver disease progression (**Table 2**). Conversely, miRNA-182 and miRNA-4485 were relatively down-regulated ( $\leq -2$  fold) in chronic HBV with progression (**Table 2**). These miRNAs might therefore be involved in HBV progression; however, their mechanism of action remains to be elucidated.

Aberrant expression of 5 miRNAs reported in this study (miRNA-3615, miRNA-342, miRNA-339, miRNA-182, and miRNA-4485) has been previously reported in relation to liver cirrhosis or HCC malignancy<sup>(29)</sup> as well as some other cancers such as lung adenocarcinoma<sup>(30)</sup>, melanoma<sup>(31)</sup>, colorectal cancer<sup>(32)</sup> (33), gastric cancer<sup>(34)</sup> (35), intrahepatic cholangiocarcinoma<sup>(36)</sup>, colon cancer<sup>(37)</sup>, and breast cancer<sup>(38)</sup> (39). Recently Wojcicka *et al.* employed next generation sequencing to investigate the expression of microRNA in tissue samples of HBV/HCV infected with liver cirrhosis and HCC. Their results revealed up-regulation of miR-339, miR-3615, and miR-342 in cirrhotic liver tissue compared to non-cirrhotic liver tissue<sup>(29)</sup>. This is similar to the results in the current study as these miRNAs were up-regulated in samples of CHB with progression to liver cirrhosis compared to patients without disease progression. However, Wojcicka *et al.* also showed relative up-regulation of miR-182 in cirrhotic liver tissues, which is in contrast to our results wherein this miR-182 was down-regulated in CHB with progression. These conflicting results may be due to the differences in experimental settings such as source of the miRNA (circulatory miRNA versus miRNA in HCC cells and tissues) as well as differences in etiology of liver disease (HBV-related cirrhosis/HCC versus HBV/HCV-related cirrhosis/HCC).

Notably, our findings also revealed 3 novel miRNAs associated with HCC malignancy (miRNA-935, miRNA-4508, and miRNA-3200). To our knowledge, these miRNAs have not previously been reported in relation to HBV-associated liver disease. However, they were previously indicated as non-invasive biomarkers in gastric and breast cancers<sup>(39)</sup> (40) (41). Specifically, Yan *et al.* reported the down-regulation of miR-935 in gastric carcinoma tissue as compared to normal tissue samples as well as in gastric cell line carcinoma as compared to that of non-gastric cells<sup>(40)</sup>. In another study, Boo *et al.* showed that the up-regulation of miR-4508 is associated with breast cancer chemo-resistance and self-renewal ability<sup>(39)</sup>. This suggests that the novel miRNAs identified in this study may serve as potential prognostic biomarkers in HBV progression.

Aberrant expression of miRNAs has been reported in a number of cancers and deregulated miRNAs have been confirmed to play vital roles in cancer initiation and development by regulating the expression of numerous tumor suppressor genes or oncogenes. For example, it has been demonstrated that miR-342 acts as a tumor suppressor gene; thus, its expression is increased during tumorigenesis<sup>(42)</sup>. It has also been identified

that tumorigenesis depends on angiogenesis, the process of new blood vessel formation from an existing vasculature<sup>(43)</sup>. For this purpose, endothelial cells undergo changes from a resting state to a rapid growth state. This change depends on some positive regulators of angiogenesis such as transforming growth factor beta (TGF $\beta$ ) and vascular endothelial growth factor (VEGF)<sup>(44)</sup>. The change may also involve the down-regulation of endogenous inhibitors of angiogenesis<sup>(45)</sup>. The eventual result is an alteration in the net balance between negative and positive regulators. Accordingly, it has been reported that miRNA-342 regulates angiogenesis likely through the modulation of TGF- $\beta$  signaling as mediated by VEGFR and endoglin, a co-receptor of the TGF- $\beta$  receptor signaling pathway<sup>(42)</sup>. Together, these findings suggest that miRNA-342 suppresses angiogenesis. Furthermore, a prior study has confirmed that miRNA-182 constitutes a prominent regulator of cancer associated processes, with supporting effects in both angiogenesis and cell proliferation<sup>(46)</sup>. In particular, using normal and HCC liver tissue, Wang *et al.* have reported that miRNA-182 stimulates HCC metastasis by preventing the expression of metastasis suppressor 1<sup>(47)</sup>.

Recent evidence has indicated the association between miRNAs and their targeted genes in various cancer-related diseases. For example, the impact of miRNA-342 on targeted genes including fatty acid synthase (FASN) and 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) was reported in prostate cancer<sup>(48)</sup>. It has been demonstrated that miRNA-342 controls lipogenesis and cholesterol synthesis in prostate cancer cells by preventing Sterol regulatory element-binding protein 1 and 2 expression as well as down-regulating FASN and HMGCR<sup>(48)</sup>. Another study has verified that miRNA-182 represents an important factor involved in HCC development and plays a vital role as an upstream regulator of the CCAAT/enhancer binding protein alpha (C/EBPA) pathway in humans<sup>(49)</sup> by directly suppressing CEBPA to control the proliferation of tumor cells. Notably, this role of miRNA-182 on CEBPA has also been reported in some tumor diseases such as lung cancer and breast cancer<sup>(49)</sup>. However, the identification of aberrantly expressed microRNAs and their target genes in HBV-associated cirrhosis/HCC remained at a preliminary stage as most prior miRNA expression studies compared patients infected with HBV or those with HCC and healthy subjects. Thus, although HBV miRNA profiling has been utilized in many studies, few have been performed with regard to the progression of HBV infection and these have been limited to studies primarily involving liver tissues (*in vivo*) and HepG2 (human liver cancer cell line) cells (*in vitro*)<sup>(20)</sup> (22) (23).

It should be noted that the participants of this study were Chinese men; thus, these results need to be replicated in women as well as in other ethnicities. In addition, our microarray may need to be validated by another method such as quantitative reverse transcription polymerase chain reaction amplification. Considering that miRNAs in general appeared to exhibit both oncogenic and tumor suppressor roles in HCC tumorigenesis, further investigation using a larger sample size is needed to provide additional evidence for these to be used as potential biomarkers for diagnosis and progression of the disease.

In conclusion, our results suggest that miRNA-935, miRNA-342, miRNA-339, miRNA-4508, miRNA-3615, miRNA-3200, miRNA-182, and miRNA-4485 may be useful as non-invasive markers to identify patients with CHB who are at risk of progression to cirrhosis and HCC. The discovery of an effective and reliable tool for early diagnosis of cirrhosis and HCC would play an essential role in the management of patients with chronic HBV infection.

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#### Conflicts of interest

The authors declare that there is no conflict of interests.

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