Expression and significance of urinary microRNA in patients with chronic hepatitis B

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Abstract
The aim of this study was to investigate the alterations of urinary microRNA (miRNA) expression and explore its clinical significance in patients with chronic hepatitis B (CHB).

The expression levels of urinary miRNA were detected by miRNA microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR) from 106 CHB and 40 healthy controls (Ctrl) subjects. The correlation between the levels of miRNA expression and clinical characteristics were analyzed. Receiver-operator characteristic (ROC) curves were generated to determine the specificity and sensitivity of each individual miRNA. MiRNAs expression were further measured by PCR from exosomes, which were isolated from urine samples. LX2 cells were transfected with miRNA inhibitor and accumulation of cytoplasmic lipid droplets was analyzed by Oil Red O staining.

miRNA expression profile analysis showed that 22 miRNAs were upregulated and 55 miRNAs were downregulated in CHB patients compared with Ctrl subjects (fold-change>1.5 and P<.05), miR-92b-3p, miR-770-5p, miR-519d-5p, and miR-7855-5p were significantly higher (P<.0001) in CHB subjects than in Ctrl subjects, ROC curve analysis showed that these four miRNAs were sensitive and specific enough to distinguish CHB and Ctrl subjects. The levels of miR-92b-3p expression were negatively correlated with total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and APOA-1. Moreover, in vitro experiments indicated that inhibition of miR-92b-3p increased lipid droplet formation in LX2 cells.

Aberrant expression of miRNAs has been observed in urine of CHB patients. Our findings may provide novel insights into the pathogenesis of CHB and may assist in the diagnosis of patients with CHB.

Abbreviations: CHB = chronic hepatitis B, Ctrl = healthy controls, LDL-C = low-density lipoprotein cholesterol, miRNA = microRNA, qRT-PCR = quantitative reverse transcription polymerase chain reaction, ROC = receiver-operator characteristic (ROC), TC = total cholesterol.

Keywords: chronic hepatitis B, expression, significance, urinary microRNA

1. Introduction
Chronic hepatitis B virus (HBV) infection represents a serious public health problem and is a leading cause of liver cirrhosis and hepatocellular carcinoma (HCC).[1,2] Up to 40% of patients with chronic hepatitis B (CHB) progress to chronic end-stage liver disease or HCC during their lifetime.[3] In China, HBV infection is a predominant etiological factor of liver disease, whereby 7.8% of the population are HBV carriers.[4] Additionally, China is an area with the highest prevalence of HCC due to the significant number of chronic HBV carriers.[5] Thus, prevention, diagnosis and treatment progress of CHB needed to be investigated and monitored.

MicroRNAs (miRNAs) are small, noncoding RNAs that play an important role in regulating various biological processes through their interaction with cellular messenger RNAs. It has been reported that miRNAs are expressed in all animal cells and have fundamental roles in cellular activities, such as the development, cellular differentiation, proliferation, apoptosis, cell-cycle control, metabolism, and cancer. Extracellular miRNAs in circulation, saliva, and urine have recently been shown to be associated with various pathological conditions,[6–10] including liver disease.[10–13] For example, a serum miRNA signature is associated with the immune control of HBV infection.[11] Serum miR-210 can be used as an indicator of HBV replication and translation, and a potential marker of necroinflammation in patients with CHB.[12] Expression of miR-155 is downregulated in peripheral blood mononuclear cells of patients with CHB.[13]
Our previous study revealed a five-miRNA profile (miR-122, -572, -575, -638, and -744) in serum that was deregulated in patients with CHB. The levels of these miRNAs can specifically predict liver injury in CHB.\(^{[9,10,14]}\) Comprehensiveness of these factors will help diagnosis, select optimal therapy and shed light on the underlying mechanism of CHB.

Urine is one of the most readily accessible biological fluids, and it harbors numerous biologically significant miRNAs. Recent studies have shown that urinary miRNAs exist in a stable form and are associated with liver disease.\(^{[9,10,14]}\) For example, urine and plasma miRNAs appear to be used as biomarkers of kidney or liver damage.\(^{[9]}\) Urinary miR-192 and -21 can be used as potential indicators for liver fluke-associated cholangiocarcinoma risk group.\(^{[10]}\) Urinary miRNAs (miR-625, -532, -618, -516, and -572) in serum that was deregulated in patients with CHB and underscored the potential importance of miRNAs in the pathogenesis of CHB.\(^{[14]}\) These miRNAs can be used as molecular biomarkers to monitor liver injury in CHB. \(^{[6]}\) Comprehensiveness of those miRNAs may serve as biomarkers for screening high-risk patients for the early detection of hepatocellular carcinoma.\(^{[14]}\)

Blood and urine are the most common types of samples employed for monitoring disease-related biomarkers.\(^{[6–14]}\) However, urine sample is more preferable as it enables non-invasive monitoring of physiological or pathological conditions.\(^{[9,10,14]}\) So far, however, miRNA profiling of the urine of CHB patients is incomplete. The goal of this study is to investigate whether urinary miRNAs can be used as molecular biomarkers to monitor the pathological development of CHB. In this study, we collected urine samples from patients with CHB and from healthy volunteers. We described the global miRNA expression profile in urine and identified a urinary miRNA signature for CHB. Our study revealed that a number of urinary miRNAs were differentially expressed in patients with CHB and underscored the potential importance of miRNAs in the pathogenesis of CHB.

### 2. Material and methods

#### 2.1. Study subjects and clinical parameters

Urine samples were collected from subjects, including 106 CHB and 40 healthy controls (Ctrl). The profiles of urinary miRNA expression were first generated with urine samples from 6 patients with CHB and 5 Ctrl subjects by microarray analysis. The levels of several miRNAs were further quantitated by real-time reverse transcription polymerase chain reaction (qRT-PCR) with urine samples from another 100 CHB patients and 35 Ctrl subjects. The diagnostic criteria for CHB followed the guidelines defined by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases in 2005.\(^{[13]}\) The diagnostic criteria for CHB include a history of hepatitis B or positive HBsAg for at least 6 months and the continuing presence of HBsAg and/or HBV DNA in the serum. The diagnosis of HBV infection is based on serologic markers, virologic markers, liver function test, biochemical assays, as well as other clinical and supportive findings. Urinary Ctrl samples were randomly selected from a collection of 40 individuals who underwent an annual physical examination at Shanghai Shuguang Hospital in Shanghai, China. Normal Ctrl were healthy subjects without medical disease. Samples of CHB were from patients seeking treatment in Shanghai Shuguang Hospital. The clinical parameters of these patients are given in Table 1. This study was subject to approval by the Institutional Review Board of Shanghai Shuguang Hospital (2012–206–22–02). An informed consent form was issued and signed by each of the participants, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki (1964).

| Parameters | CHB | Healthy |
|------------|-----|---------|
| Individuals (n) | 100 | 35 |
| Male | 84 | 25 |
| Female | 16 | 10 |
| Age (years) | 30.0 ± 15.3 | 25.4 ± 10.4 |
| ALT (IU/L) | 64.2 ± 84.0 | 21.6 ± 19.3 |
| AST (IU/L) | 48.5 ± 39.2 | 23.4 ± 20.6 |
| GGT (IU/L) | 47.7 ± 53.8 | 21.0 ± 11.7 |
| ALP (IU/L) | 86.0 ± 23.1 | 59.4 ± 20.3 |
| TBL (µm/L) | 17.9 ± 7.6 | 16.2 ± 5.8 |
| HBV DNA | 11720454.3 | 0 |
| HBV status (n) | | |
| HBsAg+ | 100 | 0 |
| HBsAg− | 0 | 35 |

The values of alanine aminotransferase (ALT), aspartate aminotransferase (AST), Gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin (TBL), and hepatitis B virus (HBV) DNA are shown as medians (range).

| HBsAg | HBsAg+ | HBsAg− |
|-------|--------|--------|
| Healthy | 35 | 0 |
| CHB | 0 | 100 |

#### 2.2. Urine samples collection and RNA isolation

All urine samples were collected in the morning and stored at −80°C. Urine total RNA was extracted from 800 µL urine samples using a mirVana PARIS kit (Ambion, Austin, TX) according to the manufacturer’s protocol and then eluted from the membrane into a collection tube with 35 µL elution buffer.

#### 2.3. Urine miRNA profiling and data analysis

The profiles of urine miRNAs from 6 CHB patients and 5 Ctrl cases were generated using Agilent Human miRNA microarray V21.0 (Agilent Technologies, Santa Clara, CA). The microarray chip consists of 2549 different probes for human miRNAs. One hundred ng RNA was used for each array. The arrays were read using an Agilent microarray scanner, and the data were extracted using Feature Extraction V10.7 (Agilent Technologies, Santa Clara, CA). All data were converted to Log Base 2. Statistical analysis of the microarray data was carried out using the Shanghai Biochip Co., Ltd. (Shanghai, China) Analysis System (SAS) (http://www.ebioservice.com). The core arithmetic of the SAS system is R, which is an open source programming language and software environment for statistical computing and graphics. Only those miRNAs with a fold difference ≥1.5 and a \(\Delta\Delta^{\text{CT}}\) value < .05 were considered significant.

#### 2.4. Quantitative real-time RT-PCR analysis

qRT-PCR-based quantification of miRNAs was performed with Bulge-Loop miRNA qPCR Primer and SYBR Green PCR Master Mixture (Toyobo, Ltd., Osaka, Japan), according to the manufacturer’s instructions, using the ABI StepOne Plus Real-Time PCR System with results normalized to U6 expression.\(^{[19,16]}\) The \(\Delta\Delta^{\text{CT}}\) method was used to calculate relative expression. Primer sequences used for RT-PCR are shown in Table 2.

#### 2.5. Urinary exosome isolation and electron microscopy

Overnight urine collections (~50 mL/subject) were obtained from all recruited subjects. Urine was precleared by both centrifugation
Table 2  
Primer sequences for qRT-PCR.

| Name              | Sequence                           |
|-------------------|------------------------------------|
| miR-92b-3p-RTP     | CACAATGGTGGTGATGCACTTTGGAAGGGCAC   |
| miR-92b-3p-RP      | ACCACTGGATGATGCACTTTGGAAGGGCAC     |
| miR-770-5p-RTP     | CACAATGGTGGTGATGCACTTTGGAAGGGCAC   |
| miR-770-5p-RP      | ACCACTGGATGATGCACTTTGGAAGGGCAC     |
| miR-5196-5p-RTP    | CACAATGGTGGTGATGCACTTTGGAAGGGCAC   |
| miR-5196-5p-RP     | ACCACTGGATGATGCACTTTGGAAGGGCAC     |
| miR-7855-5p-RTP    | CACAATGGTGGTGATGCACTTTGGAAGGGCAC   |
| miR-7855-5p-RP     | ACCACTGGATGATGCACTTTGGAAGGGCAC     |
| miR-7855-5p-FP     | ATAGGGCTGAGCTGGGATGCACTTTGGAAGGG   |
| miR-7855-5p-URP    | ATAGGGCTGAGCTGGGATGCACTTTGGAAGGG   |

*FP = forward primer, RP = reverse primer, RTP = RT primer, URP = universal reverse primer.

(300 g 10 min at 4 °C and 17,000 g 20 min at 4 °C) and filtration (0.8 μm) to remove cellular debris. Then, exosomes were isolated by two consecutive ultracentrifugation steps (200,000 g 75 min at 4 °C; 70.1 Ti rotor, Beckman Instruments, Fullerton, CA), as previously described. Pellets were suspended in phosphate buffer, and exosome quality and purity were assessed by electron microscopy.

2.6. Cell culture

Human LX2 cells (a generous gift from Dr. Xu L) were maintained in plastic culture plates in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 100 U/ml penicillin G sodium salt and 100 μM streptomycin sulfate (Gibco, Carlsbad, CA), and incubated at 37 °C under an atmosphere of 5% CO2.

2.7. Transfection of miRNA inhibitor and Oil Red O staining

The miR-92b-3p inhibitor (anti-miR-92b-3p) and miRNA inhibitor negative control (anti-miR-Con) were transfected into cells at 100 nmol/L concentrations with Lipofectamine 3000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After transfection for 24 hours, cells were treated with 10 ng/mL TGF-β1 (R&D Systems, Shanghai, China) for 48 hours. The cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde. Oil Red O (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was added, washed away, and lipid droplets were photographed. Photographs were taken at 200× magnification.

2.8. miRNA target prediction and enrichment information

The miRNA potential target genes were shown as the intersection of TargetScan (http://www.targetscan.org/), PITA (https://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), and microRNA.org (http://www.microrna.org/microrna/home.do) databases (GeneSpring software version 12.5). These putative target genes were subjected to Gene Ontology (GO) biological process annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the DAVID online analysis tool (http://david.abcc.ncifcrf.gov/). The P-value was calculated with a unilateral Fisher’s exact test and corrected by false discovery rate (FDR). GOs and pathways with P-value < 0.05 and FDR < 0.05 were considered significant.

2.9. Statistical analysis

Comparisons between groups were analyzed using the Mann-Whitney U test or Pearson correlation analysis wherever appropriate. Receiver-operator characteristic (ROC) curves were generated to evaluate the difference in the levels of urinary miRNAs between CHB and Ctrl subjects. All tests were two-tailed, and P < 0.05 was considered statistically significant.

3. Results

3.1. Aberrant miRNA expression profiling in CHB urine

To assess differential urinary miRNA expression profiles between CHB and Ctrl, miRNA microarray experiments were conducted on the total RNA obtained from urinary samples from 6 CHB and 5 Ctrl cases. Among the 2549 miRNAs analyzed, 77 were differentially expressed between CHB and Ctrl subjects. Compared with Ctrl subjects, CHB patients showed 22 upregulated miRNAs and 55 downregulated miRNAs (fold-change > 1.5 and P-value < 0.05) (Figure 1 and Table 3). For example, miR-7855-5p, -770-5p, -5196-5p, and -92b-3p were upregulated in CHB patients; miR-642a-3p, -320c, and -3138 were downregulated in CHB patients (Table 3).

3.2. Validation of urinary miRNA expression by qRT-PCR

To validate these microarray-generated results, we prepared RNA from urinary samples of another 100 CHB patients and 35 Ctrl and were subsequently subjected to qRT-PCR to measure the levels of miR-7855-5p, -770-5p, -5196-5p, and -92b-3p. We selected these four miRNAs for further analysis due to its elevated miRNA expression with a low P-value in CHB patients compared with Ctrl subjects (Table 3), and its high expression abundance in the urine based on microarray data. Identical to what we observed with microarray analysis, the levels of miR-7855-5p, -770-5p, -5196-5p, and -92b-3p were significantly higher in the urine of CHB patients than in Ctrl subjects (P < 0.0001, respectively) (Figure 2).

3.3. Levels of urinary miRNAs can be used to distinguish CHB patients from Ctrl subjects

To determine whether the levels of urinary miRNAs can be used to distinguish patients with CHB from Ctrl, we generated ROC curves to analyze the difference in the levels of urinary miR-92b-3p, -770-5p, -5196-5p, and -92b-3p between groups. Comparing CHB subjects with Ctrl, ROC curve areas of miR-92b-3p, -770-5p, -5196-5p, and -92b-3p were found to be 0.89 (95% CI: 0.84-0.94), 0.92 (95% CI: 0.88-0.97), 0.76 (95% CI: 0.67-0.85), and 0.82 (95% CI: 0.72-0.91), respectively. The sensitivity and the specificity of each of these miRNAs were 86% and 91.4%, 93% and 90.9%, 80% and 91.4%, 83% and 80% in CHB and Ctrl subjects, respectively (Figure 3). These results clearly showed that the levels of urinary miR-92b-3p, -770-5p, -5196-5p, and -7855-5p can distinguish patients with CHB from Ctrl.

3.4. Expression of urinary miRNAs in exosomes

To investigate the distribution of the urinary miRNAs, we isolated exosomes from urine samples of 6 patients with CHB and 6 Ctrl subjects. A typical electron micrograph of negatively stained urinary vesicles (Figure 4A and B) illustrates that the distribution of vesicle size shows a range of 30-100 nm. The levels of miR-92b-3p,
-770-5p, -5196-5p, and -7855-5p were further measured by PCR. We concluded that miR-92b-3p, -770-5p, -5196-5p, and -7855-5p were observed at high abundance in urinary exosomes. These results indicate that urine miRNAs such as miR-92b-3p, -770-5p, -5196-5p, and -7855-5p are likely to be mainly contained in exosomes. Urinary miRNAs could possibly be applied in the diagnosis of CHB as a novel urine-based biomarker.

3.5. Aberrant levels of urinary miR-92b-3p, -770-5p, -5196-5p, and -7855-5p correlate with clinical pathological parameters

We analyzed the potential correlations between each of these four miRNAs (miR-92b-3p, -770-5p, -5196-5p, and -7855-5p) and each of the clinical pathological parameters evaluated. We correlated the expression levels of these four miRNAs with
clinical indicators of HBV infection, including serum ALT, AST, HBsAg, HBeAg level, and HBV DNA level. However, no significant correlation was discovered (data not shown). A Pearson correlation analysis showed that correlation only existed between selected miRNAs and selected liver function parameters (Table 4). For example, miR-770-5p was significantly correlated with serum Total protein (TP) \( (r = 0.339, P = .001) \). miR-92b-3p was significantly correlated with Total cholesterol (TC) \( (r = 0.263, P = .008) \), low-density lipoprotein cholesterol (LDL-C) \( (r = 0.241, P = .016) \), and APOA-1 \( (r = 0.221, P = .028) \) (Table 4). In this study, we found that serum levels of TP were higher in CHB group than in the Ctrl group; TC, LDL-C, and ApoA-1 were lower in CHB patients than in matched Ctrl (Table 5).

### 3.6. Inhibition of miR-92b-3p increases lipid droplet formation in LX2 cells

Hepatic stellate cells (HSCs) play an important role in liver physiology and under healthy conditions they have a quiescent and lipid-storing phenotype. To further investigate whether miR-92b-3p is involved in the lipid metabolism, Human HSC (LX2) cells were transfected with miR-92b-3p inhibitor and miRNA negative control for 24 hours and treated with TGF-β1 (10 ng/mL) for 48 hours. Oil Red O staining analysis demonstrated that downregulation of miR-92b-3p allows HSCs to restore their ability to accumulate cytoplasmic lipid droplets (Figure 4C and D). Our results suggest that miR-92b-3p contribute to the regulation of lipid metabolism.

### 3.7. GO terms and KEGG pathway annotation of miR-92b-3p targets

To investigate the potential function of miR-92b-3p in lipid metabolism, we predicted its targets using TargetScan, PITA, and microRNA.org databases. There were 2430 putative targets for TargetScan, 325 putative targets for PITA, and 2466 putative targets for microRNA.org. We calculated 144 target genes by computing the intersection of the three databases (Figure 5A, Supplementary Table 1, http://links.lww.com/MD/D232). Gene ontology analysis showed that the target genes of miR-92b-3p played a part in lipid metabolism, such as negative regulation of lipid storage (ITGB3; ITGAV) and liver development (MKL2; NOTCH1; AACS; MAN2A1; JARID2) (Figure 5B). Pathway enrichment analysis showed that the target genes of miR-92b-3p...
involved in pathways related to lipid metabolism, such as the Thyroid hormone signaling pathway (ITGB3; NOTCH1; NCOA1; ITGAV), Notch signaling pathway (NOTCH1; DTX2), Hedgehog signaling pathway (BTRC; RAB23), and FoxO signaling pathway (NLK; FOXG1; RAG1) (Figure 5C). This finding clearly indicates that these miRNAs may be actively secreted and involved in CHB pathogenesis.

4. Discussion

Recent studies have shown that miRNA expression patterns are disease and tissue specific. MiRNAs are abundant in the liver and modulate a diverse spectrum of cellular processes associated with liver injury such as inflammation, apoptosis, and hepatocyte regeneration. Deregulation of miRNA expression may be a key pathogenic factor in various liver diseases, including viral hepatitis, liver fibrosis/cirrhosis, HCC, metabolic, and acute liver diseases. A clearer understanding of the mechanisms involved in miRNA deregulation would offer new diagnostic and therapeutic strategies to address liver diseases.

Cell-free miRNAs are shielded from degradation of RNase through packaging in exosomes, microvesicles and apoptotic bodies or formation of protein-miR complexes with argonaute 2 (Ago2) or high-density lipoprotein (HDL)-associated protein. Cell-free miRNAs in serum and urine are a novel class of noninvasive disease biomarker with specificity, stability and reproducibility. miRNA expression profiles are altered in various hepatic diseases compared with those in healthy conditions. For example, miR-122 was previously described to be an abundant, liver-specific miRNA. In rodents, liver injury induced by alcohol or chemicals increases the level of serum or plasma miR-122, and this increase occurs earlier than the increase in ALT, a commonly used marker.

Moreover, the level of plasma miR-122 exhibits an excellent correlation with the necro-inflammatory activity of HBV and HCV infection. Circulating miR-122 is a predictor for virological response in CHB patients with high viral load treated with nucleos(t)ide analogs. MiR-122 inhibits HBV replication through NDRG3 and CCNG1-modulated P53 activity. Serum miR-210 can be used as an indicator of HBV replication and translation, and a potential marker of necroinflammation in patients with CHB. HBV infection suppresses the expression of inflammatory macrophage miR-210. Circulating miR-210 and -22 combined with ALT can predict the virological response to interferon-alpha therapy of CHB. Zhang et al found that miR-210 negatively regulates HBV replication by targeting the HBV pre-S1 region in HepG2 cells under normoxic conditions.

Serum miR-125b was correlated with HBV
replication and liver necroinflammation, and miR-125b levels predict virologic response to nucleos(t)ide analogue treatment in patients with HBeAg-positive CHB. MiR-125b inhibits HBV expression through targeting of the SCNN1A and miR-125b also inhibits the secretion of HBsAg and HBeAg. Serum miR-146a-5p was highly expressed in HBV-infected patients. In HBV-infected hepatocytes, NF-κB-mediated miR-146a-5p induced autophagy via the miR-146a-5p-XIAP-MDM2/p53 pathway, leading to HBV propagation. Wang et al demonstrates that miR-146a upregulation in CHB causes impaired T cell function by targeting Stat1, which may contribute to immune defects and immunopathogenesis during chronic viral infection. Our previous study has revealed that serum levels of miR-122, -572, -575, -638, and -744 are deregulated in patients with CHB or nonalcoholic steatohepatitis (NASH). The levels of these miRNAs may be used as potential biomarkers for liver injury caused by CHB and NASH.

However, the complete urinary miRNA profile of CHB remains largely unknown. In the present study, using miRNA microarray, we investigated the global expression profiles in urine from CHB patients. The results revealed significant differences in expression profiles of miRNAs between CHB and Ctrl in which 22 were upregulated and 55 downregulated in CHB subjects (fold-change >1.5 and \( P < .05 \)) (Figure 1 and Table 3). Overall miRNA expression levels were downregulated in CHB patients. This change may be attributed to inhibition of miRNA expression and dysfunction of organism-specific immune response resulting from HBV infection. Subsequent validation work found that the median levels of miR-92b-3p, -770-5p, -5196-5p, and -7855-5p were significantly higher \( (P < .0001) \) in CHB than in Ctrl (Figure 2). ROC curve analysis showed that the levels of miR-92b-3p, -770-5p, -5196-5p, and -7855-5p in urine were sensitive and specific enough to distinguish CHB and Ctrl subjects. Therefore, this study has revealed a novel phenomenon of the aberrant expression levels of miRNA in urine of CHB patients.

Urine has very interesting features as a source of biomarkers; it is easy to collect, contains both proteins and metabolites, and contains extracellular vesicles (uEVs). These biomarkers are mostly derived from renal and urethral cells but may also be

Figure 3. Receiver-operating characteristic (ROC) curves for miR-92b-3p, -770-5p, -5196-5p, and -7855-5p to discriminate CHB patients from Ctrl subjects. (A) miR-92b-3p (CHB/Ctrl); (B) miR-770-5p (CHB/Ctrl); (C) miR-5196-5p (CHB/Ctrl); (D) miR-7855-5p (CHB/Ctrl). AUC = area under the ROC curve. CHB = chronic hepatitis B, Ctrl = healthy control.
directly filtered from systemic circulation. MiRNAs released from the liver of patients with CHB are likely to be affected by a variety of different mechanisms. First, miRNA levels of the liver cells could be altered, leading to changes in the number of miRNAs released via normal processes. Second, normal miRNA release from cells via microvesicles, exosomes, or protein-bound complexes could be altered. Third, cell injury and death (e.g., necrosis or apoptosis) could lead to gross leakage of miRNA. All of these processes could affect the number of miRNAs released from the liver into the plasma and, subsequently, into other cell-free body fluids, such as urine. To determine the distribution of miR-92b-3p, -770-5p, -5196-5p, and -7855-5p in urine, we also measured the levels of total RNA in the urinary exosomal enrichment fraction in 6 CHB patients and 6 Ctrl subjects. miR-92b-3p, -770-5p, -5196-5p, and -7855-5p were observed at high abundance in urinary exosomes. These results indicate that urine miRNAs, such as miR-92b-3p, -770-5p, -5196-5p, and -7855-5p, are likely to be mainly contained in exosomes. Therefore, we speculate that these miRNAs may be actively secreted and play important roles in process of chronic HBV infection.

Table 5
Coeficient of Pearson correlation between miRNA variables and clinical parameter variables (all CHB samples).

| Variables        | miR-92b-3p | miR-770-5p |
|------------------|------------|------------|
| miR-92b-3p       | 1.000      | -0.027     |
| miR-770-5p       | -0.027     | 1.000      |
| TP               | 0.086      | -0.329**   |
| TC               | -0.263*    | -0.145     |
| LDL-C            | -0.241*    | -0.106     |
| APOA-1           | -0.221*    | -0.186     |

Data are shown as the mean±SD. P<.05 miRNA vs miRNA, miRNA vs clinical parameter (2-tailed test). LDL-C=low-density lipoprotein cholesterol, TC=total cholesterol, TP=total protein.
We analyzed the potential correlations between miR-92b-3p, -770-5p, -5196-5p, -7855-5p and each of the clinical pathological parameters. However, no significant correlation was found between HBV infection and these miRNAs (data not shown).

This study shows that the expression level of urinary miR-770-5p is significantly negatively correlated with serum total protein (TP) in patients with CHB (Table 4). Serum levels of TP were higher in the CHB group than in the Ctrl group (Table 5). Notably, Zhang et al reported that serum levels of TP were lower in the CHB group than the healthy control group,[46] but were higher in the HBV-associated cirrhosis group than in the HBV-associated HCC group.[47]

The liver plays a vital role in lipid metabolism; hence, its consequent degeneration following chronic hepatitis B infection could potentially provoke dyslipidemia.[48–51] Our study revealed significantly lower TC and ApoA-1 in CHB patients than in matched Ctrl (Table 5). Some studies have also reported significantly lower TC in CHB patients than in Ctrl.[27,46,47] Expression of ApoA-1 mRNA and protein levels, as well as serum ApoA-1 levels, were lower in CHB patients than in correspondin Ctrl.[47,53] Recent evidence suggests that miRNAs are critical regulators of lipid synthesis, fatty acid oxidation, and lipoprotein formation and secretion. For example, miR-122-deletion in the whole body or specifically in the liver resulted in a marked decrease in total serum cholesterol and triglyceride (TG) levels. Anti-miR-122 therapy resulted in a significant reduction (25%–30%) of circulating cholesterol levels.[54–56] miR-27a and -27b have been shown to target RXRa and regulate fat metabolism.[57]

A growing body of evidence suggests that miR-92b-3p is involved in various human diseases.[58–61] miR-92b-3p promotes neurite growth and functional recovery via the PTEN/AKT pathway in acute spinal cord injury.[58] miR-92b-3p has been reported to act as an oncogene that promotes the colorectal carcinoma, pancreatic cancer, and gastric cancer by targeting FBXW7,[59] Gabra3[60] and HOXD10.[61] Uotani et al reported that circulating mir-92b-3p as a novel biomarker for monitoring of synovial sarcoma.[62] However, the relationship between mir-92b-3p and CHB remains unclear. We found, for the first time, that the expression level of urinary miR-92b-3p is significantly negatively correlated with serum TC, LDL-C, and APOA-1 (Table 4), implicating regulatory functions in lipid homeostasis.

HSCs play an important role in liver physiology and under healthy conditions they have a quiescent and lipid-storing phenotype. Interestingly, the inhibition of miR-92b-3p in human hepatic stellate LX2 cells displayed an increased number of Oil red O-stained lipid droplets (Figure 4C and D), suggesting that miR-92b-3p may be involved in the regulation of fat metabolism. To further investigate the function of miR-92b-3p, we predicted its potential targets using bioinformatics tools. Gene Ontology and pathway enrichment analysis showed that the target genes of miR-92b-3p played a part in lipid metabolism, such as negative correlation with lipid-related pathways.
regulation of lipid storage (TGB3; ITGAV), liver development (MKL2; NOTCH1; AACS; MAN2A1; JARID2), Thyroid hormone signaling pathway (ITGB3; NOTCH1; NCOA1; ITGAV), Notch signaling pathway (NOTCH1; DTX2), Hedgehog signaling pathway (BTRC; RAB23) and FoxO signaling pathway (NLK; FOXG1; RAG1) (Figure 5B and C). This result clearly indicates that these miRNAs might play important roles in maintaining the lipid homeostatic mechanisms of chronic HBV infection and shows that they may be actively secreted. However, their mechanism of action needs to be further elucidated in order to fully understand this phenoytype.

In summary, our studies have shown aberrant expression of miRNAs in urine of CHB patients. In addition, the expression levels of urinary miR-92b-3p are negatively correlated with TC, LDL-C and APOA-1, implicating regulatory functions in lipid homeostasis. Our findings may provide novel insights into the pathogenesis of CHB and may assist in the diagnosis of patients with CHB. Nevertheless, this approach should be further validated in larger cohorts of patients, especially those with non-CHB-related disease, to assess their efficacy and potential applicability in a screening setting.

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