Global MicroRNA Expression Profiling in the Liver Biopsies of Hepatitis B Virus–Infected Patients Suggests Specific MicroRNA Signatures for Viral Persistence and Hepatocellular Injury

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Hepatitis B virus (HBV) can manipulate the microRNA (miRNA) regulatory networks in infected cells to create a permissive environment for viral replication, cellular injury, disease onset, and its progression. The aim of the present study was to understand the miRNA networks and their target genes in the liver of hepatitis B patients involved in HBV replication, liver injury, and liver fibrosis. We investigated differentially expressed miRNAs by microarray in liver biopsy samples from different stages of HBV infection and liver disease (immune-tolerant [n = 8], acute viral hepatitis [n = 8], no fibrosis [n = 16], early [F1+F2, n = 19] or late [F3+F4, n = 14] fibrosis, and healthy controls [n = 7]). miRNA expression levels were analyzed by unsupervised principal component analysis and hierarchical clustering. Analysis of miRNA–mRNA regulatory networks identified 17 miRNAs and 18 target gene interactions with four distinct nodes, each representing a stage-specific gene regulation during disease progression. The immune-tolerant group showed elevated miR-199a-5p, miR-221-3p, and Let-7a-3p levels, which could target genes involved in innate immune response and viral replication. In the acute viral hepatitis group, miR-125b-5p and miR-3613-3p were up, whereas miR-940 was down, which might affect cell proliferation through the signal transducer and activator of transcription 3 pathway. In early fibrosis, miR-34b-3p, miR-1224-3p, and miR-1227-3p were up, while miR-499a-5p was down, which together possibly mediate chronic inflammation. In advanced fibrosis, miR-1, miR-10b-5p, miR-96-5p, miR-133b, and miR-671-5p were up, while miR-20b-5p and miR-455-3p were down, possibly allowing chronic disease progression. Interestingly, only 8 of 17 liver-specific miRNAs exhibited a similar expression pattern in patient sera. Conclusion: miRNA signatures identified in this study corroborate previous findings and provide fresh insight into the understanding of HBV-associated liver diseases which may be helpful in developing early-stage disease diagnostics and targeted therapeutics. (HEPATOLOGY 2018;67:1695-1709)

Abbreviations: ALT, alanine aminotransferase; AVH, acute viral hepatitis; cDNA, complementary DNA; ECM, extracellular matrix; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IT, immune-tolerant; miR/miRNA, microRNA; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, principal component analysis; PTEN, phosphatase and tensin homolog; STAT, signal transducer and activator of transcription.

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infection resulting in a rise of alanine transaminases are poorly understood.

Current treatment of chronic hepatitis B patients is with oral nucleotide agents, interferons, or a combination of the two. However, the current approaches fall short of expected viral clearance and disease resolution in most patients. This is mainly due to limited understanding of the HBV–host interaction in different stages of infection and disease. One such host–virus interaction could be through the regulation of host microRNAs (miRNAs) by HBV in order to facilitate its replication in hepatocytes and create a microenvironment conducive for viral persistence and liver disease. (7,8)

Recent studies suggest that miRNAs are abundant in the liver, and modulate a wide range of hepatocellular functions. Further, differential expression of miRNAs has been reported for some clinically important liver diseases, suggesting a close association between their dysregulation and liver diseases. (9–12) Besides, infection with HBV could also alter the expression patterns of miRNAs in the host. (8,12) Consequently, the role of miRNAs in gene regulation and disease progression is increasingly being recognized for diagnosis and novel therapeutic strategies for many liver diseases including HCC. (11–13) However, distinctive miRNA profiles of different stages of HBV-associated liver diseases and establishing their role in disease initiation and progression have not been studied. Specific miRNA sets and target genes facilitating viral clearance could be identified by comparing patients who develop acute hepatitis B compared to those who have chronic HBV infection. Besides, the role of miRNAs in progression of hepatic fibrosis is an area of immense interest. (14) If a set of differentially expressed miRNAs in patients with early and advanced fibrosis could be identified along with their target genes, it may become possible to develop novel strategies to inhibit liver injury and regress fibrosis. Therefore, a detailed investigation on the expression of miRNAs in the liver tissue of HBV patients should provide a better understanding of the molecular mechanisms associated with viral persistence and chronic liver disease. Besides, specific miRNA signatures could be useful in treating liver diseases owing to their precision targeting in molecular networks compared to general pharmaceuticals. (15)

In the present study, we analyzed differentially expressed miRNA in the liver biopsy samples and sera of four distinct phenotypes of HBV-related liver disease: (1) chronic HBV infection with persistent normal alanine aminotransferase (ALT) and normal histology, the immune-tolerant (IT) subject; (2) chronic HBV infection with normal or raised ALT with no evidence of fibrosis; (3) acute hepatitis B patients with spontaneous recovery; and (4) patients with early (F1, F2) or advanced (F3, F4) fibrosis. By selecting distinct patient phenotypes belonging to these stages, we studied the association of HBV replication, liver injury, and liver fibrosis with differentially expressed miRNAs in the liver tissue and the downstream target genes. The miRNA gene regulatory network suggested the involvement of 17 miRNAs and 18 target genes in hepatitis B pathophysiology. However, these miRNAs exhibited a differential distribution in the patient sera.

Materials and Methods

PATIENT GROUPS

HBV-infected patients attending the Institute of Liver and Biliary Sciences, New Delhi, were enrolled...
TABLE 1. Demographic and Clinical Characteristics of the Enrolled Patients and Healthy Subjects (Unless Otherwise Indicated, All Values Are Shown as Median and Range in Parentheses)

| Parameters          | Healthy (n = 23) | AVH-B (n = 30) | IT (n = 30) | No Fibrosis (n = 60) | Early Fibrosis (n = 60) | Advanced Fibrosis (n = 60) | P*  |
|---------------------|------------------|----------------|------------|----------------------|------------------------|---------------------------|-----|
| Age (years)         | 41.00            | 44.50          | 39.00      | 35.50                | 34.00                  | 45.50                     | 0.170 |
| Male gender (%)     | 65.7             | 65.5           | 82.5       | 83.5                 | 84.5                   | 81.2                      | 0.06 |
| Total bilirubin (mg/dL) | 0.80           | 13.90          | 0.80       | 0.80                 | 0.80                   | 0.90                      | 0.00 |
| Serum bilirubin direct (mg/dL) | 0.08         | 8.65           | 0.10       | 0.10                 | 0.10                   | 0.10                      | 0.00 |
| Serum bilirubin indirect (mg/dL) | 0.75         | 5.25           | 0.70       | 0.70                 | 0.70                   | 0.70                      | 0.00 |
| Total protein biuret (g/dL) | 7.70         | 7.3            | 7.15       | 7.30                 | 7.30                   | 7.90                      | 0.20 |
| Albumin (g/dL)      | 4.50             | 3.35           | 3.93       | 4.10                 | 4.10                   | 4.05                      | 0.00 |
| GGT (IU/L)          | 16               | 6.48           | 2.84       | 3.24                 | 4.85                   | 4.43                      | 0.07 |
| ALT (IU/L)          | 32               | 1118           | 26.00      | 35                   | 71                     | 38                        | 0.00 |
| AST (IU/L)          | 20               | 594.50         | 24.95      | 26                   | 50                     | 33                        | 0.00 |
| SAP (IU/L)          | 74               | 134.0          | 54.00      | 74                   | 83                     | 80                        | 0.00 |
| GGT (IU/L)          | 16               | 69.0           | 16         | 16                   | 16.50                  | 20                        | 0.00 |
| AFP (ng/mL)         | 2.0              | 6.48           | 2.84       | 3.24                 | 4.85                   | 4.43                      | 0.07 |
| Log10 [HBsAg] (IU/mL) | 1.93-2.08      | 1.97-77.53     | 1.23-8.66  | 1.23-8.66            | 1.77-8.52              | 1.82-11.70                | 0.09 |
| Log10 [HBV DNA] (IU/mL) | 0.10-1.10      | 1.00-1.29      | 0.90-1.20  | 0.90-1.20            | 0.90-1.20              | 1.00-1.64                 | 0.65 |
| INR                 | 1.0              | 1.15           | 1.0        | 1.0                  | 1.00                   | 1.10                      | 0.09 |
| Log10 [HBeAg] (IU/mL) | 4.24          | 3.59           | 3.68       | 4.37                 | 3.87                   | 4.00                      | 0.40 |
| Log10 [HBeAg] (IU/mL) | 5.81          | 7.01           | 4.09       | 4.32                 | 4.54                   | 4.00                      | 0.00 |

*The nonparametric Kruskal-Wallis test was applied to assess significant differences at P < 0.05.

Abbreviations: AFP, alpha-fetoprotein; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; INR, international normalized ratio; SAP, serum amyloid P component.

in this study. The study protocol was approved by the ethical committee of the Institute of Liver and Biliary Sciences. Informed consent was obtained from all patients, and their detailed history was recorded. The inclusion criteria for the six study groups (Table 1) were as follows: (1) acute viral hepatitis (AVH) patients with features of acute hepatitis, ALT > 10 times the upper limit of normal, hepatitis B surface antigen (HBsAg)-positive, hepatitis B e antigen (HBeAg)-positive, immunoglobulin M antibody to hepatitis B core antigen–positive, and spontaneous viral clearance of HBsAg by 12 months of follow-up; (2) IT group, HBsAg-positive patients for >6 months, with persistent normal ALT, no fibrosis, HBeAg-positive, and elevated HBV DNA per the European Association for Study of Liver guidelines of 2012; (3) chronic HBV infection with no fibrosis, patients HBsAg-positive for >6 months with normal or raised ALT, HBeAg-positive/negative, antibody to HBeAg-positive/negative, and HBV DNA positive with F0 fibrosis score; (4) chronic HBV infection with early fibrosis (F1,F2) or (5) advanced fibrosis (F3,F4); (6) healthy controls, age-matched, sex-matched, and nutritional status–matched asymptomatic healthy individuals with no history or clinical features of any liver disease, with normal transaminases, and negative for immunoglobulin M antibody to hepatitis A virus, immunoglobulin M antibody to hepatitis E virus, HBsAg, antibody to HBeAg, and immunoglobulin G antibody to hepatitis B core antigen markers. All patient samples were collected at the time of diagnosis and before starting any treatment.

Exclusion criteria included co-infection with hepatitis C, hepatitis D, or human immunodeficiency virus infection; decompensated liver disease (defined by serum bilirubin level >2.5 times the upper limit of normal, a
prothrombin time prolonged by more than 3 seconds, a serum albumin level < 3 g/dL, or a history of ascites, variceal hemorrhage, hepatic encephalopathy, or grade III or IV esophageal varices unless banded); chronic hepatitis B with Child-Pugh B and C; evidence of liver disease due to other etiology; serum creatinine > 1.5 times the upper limit of normal; hemoglobin < 10 g/dL; platelet count < 70,000/mm³; white cell count < 3,000/mm³; serious concurrent medical illnesses like malignancy, severe cardiopulmonary disease, uncontrolled diabetes mellitus; and alcohol consumption > 20 g/day. All patients were treatment-naive at the time of sampling.

COLLECTION OF LIVER BIOPSY

A small portion of liver tissue from HBV-infected patients undergoing liver biopsy for diagnostic purposes was collected in the RNAlater stabilizing solution (Ambion, catalog no. AM7020).

ISOLATION OF TOTAL RNA AND ITS QUALITY CONTROL

Total RNA including miRNAs were isolated from liver biopsies using the mirVana miRNA isolation kit (Ambion, catalog no. AM1560) per the manufacturer's protocol. RNA was eluted in 60 μL of nuclease-free water. The concentration of RNA was measured using Nanodrop, and its quality and integrity were ascertained by the Bioanalyzer 2100 using two RNA kits, Agilent RNA 6000 Nano kit (catalog no. 5067-1511) and Agilent small RNA kit (catalog no. 5067-1548), per the manufacturer's instructions.

ANALYSIS OF miRNA EXPRESSION BY MICROARRAY

miRNA expression was determined using Agilent's human miRNA microarray version V16 which contains probes for 1,205 human and 144 viral miRNAs from the Sanger database represented by an average of 10 probes per miRNA. After washing, microarrays were scanned using Agilent's G2565BA microarray scanner, and the signal intensities were captured using Agilent Feature Extraction version 10.1 software. After quality validation, the differentially expressed microRNAs were identified using Gene Spring analysis software, version 12.0 (Agilent Technologies Inc., Santa Clara, CA). Each miRNA microarray was normalized to the 90th percentile, and interarray normalization was done to the median of all samples. Stringent quality control of each sample was done considering parameters such as internal hybridization controls, spike-in ratio, correlation coefficient among the replicates, normalization factor, saturated features, and minimum and maximum fold expression in a sample. Samples identified as outliers were removed from further analysis, and the inliers were renormalized for further downstream analysis.

BIOLOGICAL ANALYSIS OF DIFFERENTIALLY EXPRESSED miRNAs

Differentially expressed miRNAs were identified by applying a fold-change threshold of absolute fold-change ≥ 2 and a statistically significant t test P value < 0.05 by Student t test (Supporting Table S1). Unsupervised hierarchical clustering of differentially expressed genes between patient groups was done using Pearson's uncentered algorithm with the average linkage rule. Target-based pathway enrichment analysis of miRNAs was done using DIANA-miRPath v3.0 (http://www.microrna.gr/miRPathv3) with micro-T-CDS as the choice of target database with default parameters. The key nodes and edges enriched in each interpretation group were identified by BridgeIsland Software (Bionivid Technology Pvt. Ltd., India). The enrichment significance of specific biochemical and signaling pathways was visualized by clusterProfiler (https://guangchuangyu.github.io/clusterProfiler/), whereas the miRNA–mRNA gene regulatory network was visualized using CytoScape V 2.8.2.

QUANTITATIVE RT-PCR OF miRNA AND TARGET GENES

The miRNA levels in total RNA isolated from liver biopsies were measured by real-time quantitative RT-PCR. Individual miRNA LNA primer sets were procured from Exiqon (Supporting Table S2). Complementary DNA (cDNA) was synthesized by reverse-transcription using 10 ng of total RNA according to the manufacturer's instructions (Universal cDNA Synthesis kit; Exiqon, Denmark). Following first-strand cDNA synthesis, SYBR green quantitative PCR was carried out using the miRCURY LNA Universal RT miRNA PCR kit (Exiqon, Denmark) as detailed in the kit. Real-time reactions were performed in an ABI VIIA7 (Applied Biosystem, Foster City, CA). U6 RNA (Exiqon) was used as control. The relative
expression was analyzed using the ΔΔCt method.\(^{(16)}\) miRNAs from patient sera (200 μL) were isolated using the miRCURY RNA Isolation Kit–Biofluids (Exiqon, catalog no. 300112) according to the manufacturer’s instructions, quantified by quantitative RT-PCR, and analyzed as above. miR-103a (Exiqon) was used as an internal control.

mRNA levels in the liver were measured by quantitative RT-PCR using total RNA. cDNA synthesis was performed using the Verso cDNA Synthesis kit (Thermo Scientific) per the manufacturer’s instructions. Quantitative RT-PCR was performed using the DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific) and gene-specific primers from Sigma-Aldrich, India (Supporting Table S3), followed by melting curve analysis in an ABI VIIA7 (Applied Biosystem) cycler. We used 18S ribosomal RNA as an invariant control. Results were analyzed by the comparative ΔΔCt method.\(^{(16)}\)

STATISTICAL ANALYSES

For identification of differentially expressed miRNAs, the test \(P\) value (unpaired Student \(t\) test with Benjamini-Hochberg false discovery rate correction) threshold adjusted for a false discovery rate of \(<0.05\) was considered. miRNA target-based pathway analysis using miRPath was done with a \(P\) value threshold of \(<0.05\) (Fischer’s exact test) along with false discovery rate correction enabled for identifying pathways that are enriched and a micro-T threshold of 0.8 for genes targeted by differentially expressed miRNAs. Continuous variables in the demographic and clinical characteristics of patients and control subjects were summarized as median (range) and categorical variables as frequency and percentage. The nonparametric Kruskal–Wallis test was applied to assess significant differences by comparing normally distributed variables.

Results

CLINICAL CHARACTERISTICS OF THE ENROLLED PATIENTS WITH HBV INFECTION

A total of 400 patients were assessed for eligibility criteria, and 263 patients were enrolled in this study.
(Supporting Fig. S1). The demographic and clinical characteristics of the enrolled HBV patients and healthy subjects are given in Table 1. Of these, 137 patients were excluded based on poor quality of RNA isolated from their liver biopsy. Whole-genome miRNA expression profiling was done by microarray on RNA isolated from 80 subjects. The remaining 183 samples were stored for validation studies. Patients and healthy subjects were of either sex and 35-55 years of age. Serum bilirubin and albumin levels in healthy subjects and all patient groups except the AVH group were in the normal range. Serum bilirubin, ALT, and aspartate aminotransferase levels were much higher in the AVH group (Table 1).

**ANALYSIS OF DIFFERENTIALLY EXPRESSED miRNAs AND THEIR CLUSTERS**

The miRNA array data were analyzed by unsupervised principal components analysis (PCA) as well as by hierarchical clustering to distinguish (similarity versus difference) between various patient groups. Only healthy subjects and patients with advanced fibrosis formed independent clusters. Further, three of the five patient groups (early fibrosis, IT, and no fibrosis) showed close association. Patients with early fibrosis showed partial separation from the other disease groups, while those with AVH appeared as a distinct
subset. These patterns suggest that there is continuity of injury and disease progression (Fig. 1A). Further, hierarchical cluster analysis of the data substantiated our PCA findings and showed some overlap in miRNA expression among the different patient groups (Fig. 1B).

**FIG. 3.** Unsupervised clustering of most differentially expressed miRNAs compared between different HBV patient groups and healthy controls. Pearson’s uncentered algorithm with average linkage rule-based unsupervised hierarchical clustering of differentially expressed transcripts showing distinct patterns of up-regulated and down-regulated miRNAs. These miRNAs are specific to disease stage and reproducibility in their absolute expression levels within the cohort profiled. Columns represent analyzed samples, while rows represent miRNAs. The miRNA clustering tree is shown on the left, and the sample groups appear at the top. The color scale shown at the top left illustrates the relative expression level of the miRNAs, with red indicating a high expression level and green indicating a low expression level.
Next, we analyzed the distribution of differentially expressed miRNAs for specificity and commonality in various patient groups and healthy subjects. We found 15 miRNAs to be up-regulated and 12 miRNAs to be down-regulated in the IT group compared to healthy controls (Supporting Table S4). In the AVH patients, 12 miRNAs were up-regulated and two miRNAs were down-regulated in comparison to the IT group. Between the early fibrosis and no fibrosis groups, 13 miRNAs were up-regulated, while one miRNA was down-regulated. Likewise, in advanced fibrosis compared to early-stage fibrosis, 13 miRNAs were up-regulated and four miRNAs were specifically down-regulated. Further, the distribution of differentially expressed miRNAs indicated a highly disease stage–specific up-regulation and down-regulation of miRNAs, suggesting a very specific miRNA-mediated posttranscriptional gene regulation in each stage during disease progression (Fig. 2). Only three up-regulated miRNAs were common between the IT group and the healthy or AVH group (Fig. 2A, lower panel). Interestingly, except for the IT group, fewer miRNAs were down-regulated in each disease group, with virtually no overlapping expression (Fig. 2B).

**BIOLOGICAL ANALYSIS OF DIFFERENTIALLY EXPRESSED miRNAs**

The levels of differentially expressed miRNAs were analyzed by unsupervised hierarchical clustering using Pearson’s uncentered algorithm with average linkage rule (Fig. 3). The differentially expressed miRNAs showed a good separation between healthy and IT patients (Fig. 3A) as well as a partial separation between the AVH and IT groups (Fig. 3B). For example, miR-3647-5p and 766-3p, which were overexpressed in the healthy group, were specifically shut down in the IT group, whereas miRNAs expressed at low levels in the healthy group (e.g., miR-20a-3p and miR-340-3p) were up-regulated in the IT group (Fig. 3A). Similarly, miR-3613-3p and miR-129-2-3p, showing low expression in the AVH group, were up-regulated in the IT group (Fig. 3B). Importantly, no distinct miRNA expression pattern was observed when heat map data of “no fibrosis versus early fibrosis”
or “early fibrosis versus late fibrosis” were compared (Fig. 3C,D), suggesting a multifactorial etiology of liver fibrosis. Furthermore, the heat maps are in agreement with the PCA and hierarchical clustering, which also showed no clear-cut separation among the IT, no fibrosis, early fibrosis, and AVH groups. Next, using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, we performed enrichment analysis for differentially expressed miRNAs in hepatitis B patients. Significantly enriched fifteen pathways in KEGG database ($P < 0.05$) are shown in Fig. 4.

**NETWORK OF miRNAs AND THEIR TARGET GENES INVOLVED IN HEPATITIS B PATHOPHYSIOLOGY**

Next, differentially expressed miRNAs were subjected to target-based pathway enrichment analysis to identify key nodes and edges in each interpretation group, and the miRNA–mRNA gene regulatory networks were identified. The partial network clearly revealed 17 miRNAs and 18 target gene interactions (Fig. 5) with four distinct nodes, each representing a stage-specific gene regulation by miRNAs during hepatitis B–related disease progression (see below).

**miRNAs Associated With Immune Tolerance**

Levels of miR-199a-5p, miR-221-3p, and let-7a-5p were elevated in the IT patients, who did not show any discernible biochemical change or hepatocellular injury but did show active viral replication and persistence, possibly by targeting host genes important for innate immune suppression and HBV replication (Fig. 5A). Importantly, quantitative RT-PCR analysis of three miRNAs and their target genes confirmed the microarray data and revealed elevated levels of miR-199a-5p, miR-221-3p, and Let-7a-3p in the IT group compared to healthy controls, whereas their cognate target genes, *DDX3X, STAT2*, and *TBK1*, were down-regulated (Fig. 6A).
FIG. 6. Validation of expression of miRNAs and their target genes in the liver of hepatitis B patients. Expression of 17 miRNAs and their corresponding 18 target genes was analyzed by quantitative RT-PCR in the RNA samples isolated from the liver biopsies of different patient groups and healthy control (15 samples/group) to confirm the microarray data. The 18S ribosomal RNA was used as an internal control for target genes, whereas and U6 RNA was used as an internal control for miRNAs. PCR primer sequences for miRNAs and their target genes are given in Supporting Table S2 and S3, respectively. Bar charts represent quantitative RT-PCR results of miRNAs in the IT group versus healthy controls (A), the AVH-B group versus the IT group (B), the early fibrosis group compared to the no fibrosis group (C), and the advanced fibrosis group versus the early fibrosis group (D). All experiments were performed in triplicate. Data are shown as mean ± SD. *P < 0.05, **P < 0.01.
miRNAs Identified With AVH

Figure 5B shows up-regulation of miR-125b-5p and miR-3613-3p and down-regulation of miR-940 in the AVH group. Both miR-125b-5p and miR-3613-3p target signal transducer and activator of transcription 3 (STAT3) expression, required for sustaining cell proliferation, differentiation, and survival. Again, the array data were confirmed by quantitative RT-PCR, which showed elevated levels of miR-3613-3p and miR-125b-5p and down-regulation of miR-940. As expected, miR-940 target genes, such as MAVS, SMAD4, and TGFBR1, were up-regulated, whereas STAT3 expression was low for being a common target of miR-125b-5p and miR-3613-3p (Fig. 6B).

miRNAs Associated With Early Fibrosis

Liver fibrosis is apparently caused by excessive stimulation of hepatic stellate cells that produce various types of extracellular matrix (ECM) in response to transforming growth factor beta and platelet-derived growth factor. However, the miRNA regulation of early fibrosis is poorly understood. Our network analysis of up-regulation of miR-34b-3p, miR-1224-3p, and miR-1227-3p and down-regulation of miR-499a-5p in early fibrosis (Fig. 5C) could be confirmed by quantitative RT-PCR (Fig. 6C). Further, target gene expression analyses revealed enhanced expression of miR-499a-5p target genes CDKN1A and IKBKB and expected down-regulation of specific target genes in early fibrosis: ELK1 and AKT2 (for miR-1224-3p), GRB10 and PIK3CA (miR-34-3p), and HSPG2 and PTEN (miR-1227-3p) (Fig. 6C).

miRNAs Identified With Advanced Fibrosis

If hepatic injury persists, enhanced deposition of ECM proteins causes gradual substitution of liver parenchyma and distortion of hepatic architecture, called “advanced liver fibrosis.” Our network analysis identified seven miRNAs associated with advanced fibrosis. Of these, five miRNAs (miR-1, miR-10b-5p,
miR-96-5p, miR-133b, and miR-671-5p) were up-regulated; these can target the expression of some key transcription factors including ATF2, E2F3, and CREB3L2 (Fig. 5D). However, miR-20b-5p and miR-455-3p, which are known to target the cell cycle inhibitor p21CIP1 gene, were down-regulated in these patients. Further, quantitative RT-PCR analyses confirmed the miRNA levels and their cognate genes in this group (Fig. 6D).

ANALYSIS OF CIRCULATING miRNAs IN HEPATITIS B PATIENTS

As miRNAs produced in the liver could also be released in the blood circulation through extracellular vesicles and exosomes, we also measured the levels of all 17 differentially expressed miRNAs by quantitative RT-PCR in patient sera. The quantitative RT-PCR results (Fig. 7) presented a different expression pattern of miRNAs in sera. For example, miR-199a-5p and Let-7a-5p levels were low in IT patient sera (compared to healthy control), though otherwise they are elevated in liver, whereas miR-221-3p levels remained elevated as in liver (Fig. 7A). Likewise, miR-125b-5p and miR-3613-3p levels were low in the sera of AVH-B patients compared to liver, whereas miR-940 levels remained low in both compartments (Fig. 7B). Conspicuously low levels of miR-1227-3p and elevated levels of miR-499-5p were observed in sera of early fibrosis patients, while miR-34b-3p and miR-1224-3p levels remained higher as in liver (Fig. 7C). Interestingly, all seven miRNAs (miR-1, miR-10b-5p, miR-20b-5p, miR-96b-5p, miR-133b, miR-455-3p, and miR-671-5p) were high in sera of the advanced fibrosis group (Fig. 7D), though hepatic levels of miR-10b-5p, miR-20b-5p, and miR-671-5p were significantly low.

Discussion

Infection with HBV could lead to distinctly expressed miRNAs at different stages of the disease. We analyzed differential expression of 17 miRNAs in the liver biopsy samples of patients with different stages of HBV-associated liver disease. We further evaluated their potential as biomarkers in patient sera.

miRNAs ASSOCIATED WITH VIRAL REPLICATION AND VIRAL PERSISTENCE

We observed elevated levels of miR-199a-5p, miR-221-3p, and let-7a in the IT patients (Figs. 5A and 6A). miR-199a-3p is an abundant miRNA in the liver and a known regulator of some key genes required for normal liver physiology. miR-199a can down-regulate DEAD-box RNA helicase 3 and facilitate HBV replication in hepatocytes, leading to viral persistence. miR-199a-3p regulates the expression of mammalian target of rapamycin and hepatocyte growth factor receptor c-Met in HCC cells. Thus, down-regulation of both c-Met and its downstream effector ERK2 by miR-199a could be an effective strategy to inhibit cell proliferation and prevent disease progression.

miR-221-3p reportedly targets kinase TBK1, which is important for interferon production and virus clearance. As expected, down-regulation of TBK1 in the liver will promote viral persistence. Interestingly, both HBV polymerase and HBx protein are known to inhibit beta-interferon production by disrupting downstream events. Besides, miR-221 can target tumor suppressor p27 and induce liver tumorigenesis.

Let7a-5p, a tumor suppressor miRNA, was found to be up-regulated in the IT subjects. However, in cell culture, viral HBx down-regulates let-7a expression and supports cell proliferation by up-regulating STAT3. The reason for elevated levels of Let-7a in the IT patients is not clear. However, elevated Let-7a-5p levels are known to affect the innate immune response through the transcription factor STAT2 and to promote viral persistence. Further, elevated let-7a levels positively correlate with HBV replication in HCC tissues. These data suggest that elevated levels of miR-199a-5p, miR-221-3p, and let-7a-5p apparently contribute to the suppression of the innate immune response, permitting HBV replication and viral persistence. However, the circulating levels of these miRNAs were different as evident from down-regulated miR-199a-5p and Let-7a-5p levels, whereas the miR-221-3p level remained high (Fig. 7A). Thus, serum miR-221-3p could be considered a biomarker for identification of HBV-infected IT patients.

miRNAs INVOLVED IN AVH AND HEPATOCELLULAR INJURY

Our microarray data also revealed increased levels of miR-125b-5p and miR-3613-3p and down-regulation
of miR-940 in the AVH patients (Figs. 5B and 6B). Both miR-125b-5p and miR-3613-3p are well-known regulators of STAT3, which contributes to liver development and regeneration.\(^{27}\) STAT3 cooperates with transforming growth factor beta 1 to activate hepatic stellate cells and exacerbate liver injury and fibrosis.\(^{28}\) Further, ectopic expression of miR-125b is reported to both facilitate HBV replication as well as inhibit the formation HBV DNA intermediates and secretion of HBsAg and HBeAg.\(^{29,30}\) Though blockage of STAT3 is considered a therapeutic target in liver cancer, the therapeutic use of miR-125b-5p and miR-3613-3p needs further investigation.\(^{31}\) However, serum levels of miR-125b-5p as well as miR-3613-3p were low in AVH patients (Fig. 7B), suggesting their limited application as a biomarker. Interestingly, chronic hepatitis B patients show elevated plasma levels of miR-125-5p, perhaps to suppress viremia.\(^{32}\)

miR-940 is a highly expressed miRNA in stomach tissue compared to other tissues such as liver, breast, thyroid, and lung.\(^{33}\) Interestingly, in our study, miR-940 levels were down-regulated in the liver biopsies and sera of AVH patients (Figs. 6B and 7B). miR-940 is reported to inhibit the migratory and invasive potential of cells.\(^{34,35}\) miR-940 is known to engage several crucial cellular pathways such as transcription factor SMAD4, transforming growth factor beta receptor, and mitochondrial antiviral signaling protein, which functions as an adaptor for RIGI-like receptor signaling during antiviral innate immunity.\(^{36}\) Therefore, down-regulation of miR-940 is expected to attenuate antiviral mechanisms of cells and allow viral persistence.

Interestingly, miR-940 is found to be down-regulated in gastric cancer, pancreatic ductal adenocarcinoma, and HCC.\(^{37,38}\) It shows progressively lower expression in early to advanced stages of gastric cancer and thus has potential as a cancer biomarker. Therefore, it will be equally important to evaluate the diagnostic potential of miR-940 in different stages of HBV-related liver disease.

### miRNAs ASSOCIATED WITH EARLY FIBROSIS

Our miRNA network analysis identified that miR-34b-3p, miR-1224-3p, and miR-1227-3p were up-regulated in the early stage of hepatic fibrosis, while miR-499a-5p was down-regulated (Figs. 5C and 6C). There are no published reports linking the association of these miRNAs with liver fibrosis. The miR database suggested that elevated levels of miR-34b-3p should target PIK3CA and GRB10 expression, miR-1224-3p will down-regulate AKT2 and ELK1, while miR-1227-3p will target phosphatase and tensin homolog (PTEN) and heparan sulfate proteoglycan 2. A recent report suggests that hepatic stellate cells can be readily activated in the liver of PTEN null mice compared to control mouse liver. However, deletion of AKT2, the downstream target of the PTEN signal, blocks nonalcoholic steatohepatitis development and alleviates fibrosis.\(^{39}\) Further, miR-1224-3p target Elk1, which is considered an important regulator of liver regeneration.\(^{40}\) However, the biological relevance and diagnostic potential of up-regulated miRNAs in liver fibrosis remains to be established in view of the elevated levels of miR-1224-3p and miR-34b-3p in patient sera (Fig. 7C). In a recent study on 39 patients with early-stage fibrosis with HBV and hepatitis C virus infection, plasma levels of miRNA-200b and miRNA-122 were significantly up-regulated, whereas their levels were down-regulated in circulating vesicles.\(^{41}\) Therefore, it will be important to investigate whether miRNAs present in plasma or circulating vesicles have a better diagnostic potential.

In early fibrosis patients, we observed a significant down-regulation of miR-499a-5p levels in the liver, though its serum levels were elevated. miR-499a-5p is reported to facilitate the expression of kinase inhibitors cyclin-dependent kinase inhibitor 1 (or p21Cip1) and inhibitor of nuclear factor kappa-B kinase subunit beta or inhibitor of nuclear factor kappa-B kinase subunit beta. While hepatic activation of inhibitor of nuclear factor kappa-B kinase nuclear factor kappa B is considered sufficient to induce liver fibrosis by way of macrophage-mediated chronic inflammation,\(^{42}\) p21 expression is reported to be up-regulated by the stress of inflammation and fibrosis.\(^{43}\) Inhibitor of nuclear factor kappa-B kinase subunit beta is being investigated as a target for new anti-inflammatory drugs.

### miRNAs THAT COULD REGULATE HBV-INDUCED DISEASE PROGRESSION

Normally, the ratio of homeostatic hepatic cells and ECM is maintained in the liver by well-balanced synthesis and degradation of ECM components. However, in cirrhotic liver, there is an alteration in this balance due to an excessive synthesis and deposition of ECM proteins (fibrogenesis) and/or a reduction in the removal of this excess of ECM proteins (by fibrolysis),
with the consequent onset of fibrotic scarring. In the population with HBV-related cirrhosis, we identified seven unique miRNAs. Of these, five miRNAs (miR-1, miR-10b-5p, miR-96-5p, miR-133b, and miR-671-5p) were found to be up-regulated, and collectively these could regulate the expression of three important transcription factors (ATF2, E2F3, and CREB3L2) (Figs. 5D and 6D). Importantly, the expression of ATF2 target genes is frequently down-regulated in human cancers, suggesting their involvement during tumor development. Increased expression of miR-1 is significantly associated with chronic HBV infection, perhaps due to induction of impaired immune responses in these patients, while its reduced serum levels have been proposed as a good prognostic marker for HCC. It has also been suggested that elevated levels of miR-10b could serve as a noninvasive biomarker for predicting liver metastasis, while serum miR-96 could be a good biomarker for HCC patients with chronic HBV infection. On the other hand, miR-20b-5p and miR-455-3p levels were down-regulated in these patients. These miRNAs are known to specifically regulate the expression of the cell cycle regulatory p27<sup>Kip1</sup> gene, which may be involved in the regulation of cellular senescence in the diseased liver. Importantly, the sera in this patient group carried elevated levels of most microRNAs except miR-455-3p, which needs to be validated as a biomarker (Fig. 7D).

To summarize, this study demonstrates specific intracellular miRNAs and their target genes as potential biomarkers for hepatitis B–associated and stage-specific liver diseases. Future studies are required to understand the role of differentially expressed miRNAs in liver pathology in suitable models and clinical correlates of stage-specific circulating miRNAs. Regulation of miRNA activity through the systemic delivery of miRNA inhibitors or mimics could provide additional opportunities for intervening in disease processes.

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