Global micro RNA expression profiling in the liver biopsies of Hepatitis B Virus infected patients suggests specific miRNA signatures for viral persistence and hepatocellular injury

Short title: Differentially expressed miRNAs in HBV-associated liver diseases

Avishek Kumar Singh[1], Sheetalnath Babasaheb Rooge[1], Aditi Varshney[1], Madavan Vasudevan[2], Ankit Bhardwaj[3], Senthil Kumar Venugopal[1,4], Nirupama Trehanpati[1], Manoj Kumar[5], Robert Geffers[6], Vijay Kumar[1], and Shiv Kumar Sarin[1,5,*]

[1]Department of Molecular and Cellular Medicine, Institute of Liver and Biliary Sciences, New Delhi, India

[2]Bionivid Technology Private Limited, Bangalore, India

[3]Department of Clinical Research, Institute of Liver and Biliary Sciences, New Delhi, India

[4]Faculty of Life Sciences & Biotechnology, South Asian University, New Delhi, India

[5]Department of Hepatology, Institute of Liver and Biliary Sciences, New Delhi, India

[6]Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig, Germany

*Corresponding author:

Dr. Shiv Kumar Sarin, Senior Professor, Department of Hepatology

Institute of Liver and Biliary Science (ILBS)

New Delhi-110070, India

Phone: +91-11-46300000

E-mail: sksarin@ilbs.in

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Abstract

Hepatitis B virus (HBV) can manipulate the 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 the liver biopsy samples from different stages of HBV infection and liver disease [immune tolerant (IT; n= 8); acute viral hepatitis (AVH; n=8); no fibrosis (n=16); early (F1+F2) (n=19) or late fibrosis (F3+F4) (n=14) and healthy controls (n=7)]. The miRNA expression levels were analyzed by the unsupervised principal component analysis (PCA) 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 IT 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 AVH group, miR-125b-5p and miR-3613-3p were up whereas miR-940 was down which might affect cell proliferation via STAT3 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.

Conclusions: miRNA signatures identified in this study corroborate previous findings and provides fresh insights in the understanding of HBV-associated liver diseases which may be helpful in developing early stage disease diagnostics and targeted therapeutics.

Keywords: Liver fibrosis, acute viral hepatitis, RT-qPCR, Microarray, circulating miRNA, mRNA, alanine aminotransferase

Introduction

Hepatitis B virus (HBV) is a non-cytopathic, hepatotropic DNA virus that causes acute and chronic hepatitis leading to liver fibrosis, cirrhosis and to hepatocellular carcinoma (HCC).[1,2] It is estimated that nearly one-third of the world's population carries HBV infection with ~240 million people being chronically infected.[3] The host cellular mechanisms for the resolution or persistence of HBV infection remain unclear.[4] Moreover, the underlying mechanisms of cellular injury during chronic hepatitis B infection resulting in rise of alanine transaminases are poorly understood.

Current treatment of chronic hepatitis B patients is with oral nucleot(s)ide agents, interferons or a combination of the two.[5] However, the current approaches fall short of expected viral clearance and disease resolution in most patients.[7] This is mainly due to limited understanding of the HBV-host interaction in different stages infection and disease. One such host-viral interaction could be through the regulation of host micro RNAs (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 in abundance in liver to modulate a wide range of hepatocellular functions.[9] 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 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 to

different stages of HBV-associated liver diseases or establishing their role in disease initiation and progression have not been studied. Identification of specific miRNA sets and target genes facilitating viral clearance could be discerned by comparing patients who develop acute hepatitis B as compared to those who have chronic HBV infection. ~~Likewise~~ 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 as compared to general pharmaceuticals.[15]

In the present study, we have analyzed differentially expressed miRNA in the liver biopsy samples and sera of four distinct phenotypes of HBV related liver disease; namely i) chronic HBV infection with persistent normal alanine aminotransferase (ALT) and normal histology- the IT subject, ii) chronic HBV infection with normal or raised ALT with no evidence of fibrosis, iii) acute hepatitis B patients with spontaneous recovery and iv) patients with early (F1, F2) and advanced fibrosis (F3, F4). By selecting distinct patient phenotypes belonging to these stages, we have 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 in this study. The study protocol was approved by Institutional ethical committee of ILBS. Informed consent was obtained from all patients and their detailed history recorded. The inclusion criteria for the six study groups (Table 1) were as follows: i) Acute viral hepatitis-(AVH):patients with features of acute hepatitis, ALT >10x ULN, HBsAg positive, HBeAg positive, IgM Anti-HBc positive and spontaneous viral clearance of HBsAg by 12 months of follow up of the patients;(ii) immune tolerant (IT): HBsAg positive patients for more than 6 months, with persistent normal ALT, no fibrosis, HBeAg positive, and elevated HBV DNA as per the European Association for Study of Liver (EASL) guidelines of 2012; (iii) Chronic HBV infection with no fibrosis: patients with HBsAg positive for >6 months with normal or raised ALT, HBeAg positive/negative, anti-HBe positive/negative and HBV DNA positive with F0 fibrosis score; (iv) Chronic HBV infection with early fibrosis (F1,F2) or (v) advanced fibrosis (F3,F4); (vi) healthy controls: age, sex and nutritional status matched asymptomatic healthy individuals with no history or clinical features of any liver disease and with normal transaminases and negative for IgM anti-HAV, IgM anti-HEV, HBsAg, anti-HBe, IgG anti-HBc markers. All patient samples were collected at the time of diagnosis and before start of any treatment.

Exclusion criteria included co-infection with hepatitis C, hepatitis D or HIV infection; decompensated liver disease (defined by serum bilirubin level more than 2.5 times the upper limit of normal, a pro-thrombin time prolonged by more than 3s, a serum albumin level lower than 3 g/dL or a history of ascites, variceal hemorrhage or hepatic encephalopathy or Grade III

and IV esophageal varices unless banded); chronic hepatitis B with Child-Pugh B and C; evidence of liver disease due to other etiology; serum creatinine more than 1.5 times upper limit of normal; hemoglobin <10 g/dL; platelet count <70,000 per cubic millimeter; white cell count <3,000 per cubic millimeter; serious concurrent medical illnesses like malignancy, severe cardiopulmonary disease, uncontrolled diabetes mellitus, alcohol consumption more than 20 g/day].. All patients were treatment naïve at the time of sampling.

COLLECTION OF LIVER BIOPSY

A small portion of liver tissue from the HBV-infected patients, undergoing liver biopsy for diagnostic purposes, was collected in the RNAlater® (Ambion, Cat No: AM7020).

ISOLATION OF TOTAL RNA AND ITS QUALITY CONTROL

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

ANALYSIS OF miRNA EXPRESSION BY MICROARRAY

The miRNA expression was determined using Agilent´s human miRNA microarray version V16 which contains probes for 1205 human and 144 viral miRNAs from the Sanger database represented by an average of 10 probes per miRNA. After washing, the 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, USA). Each miRNA microarray was normalized to 90th Percentile and Inter array normalization was done to median of all samples. Stringent quality control of each sample was done considering parameters such as internal hybridization controls, spike-in ratio, Correlation co-efficient 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 re-normalized 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 greater than or equal to 2 and a statistically significant t test p value of <0.05 by Student's t-test (Table S1). Unsupervised hierarchical clustering of differentially expressed genes between patient groups were done using Pearson Uncentered algorithm with Average linkage rule. The 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 groups 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 (RT-qPCR) OF miRNA AND TARGET GENES

The miRNA levels in total RNA isolated from liver biopsies were measured by real time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). Individual miRNA LNA™ primer sets were procured from Exiqon (Table S2). cDNA was synthesized by reverse transcription using 10ng of total RNA according to the manufacturer's instructions (Universal cDNA synthesis kit, Exiqon, Denmark). Following first strand cDNA synthesis, SYBR green qPCR was carried out using miRCURY LNA Universal RT miRNA PCR kit (Exiqon, Denmark) as per detail of 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 $2^{-\Delta\Delta Ct}$ method. [16] miRNAs from patient sera (200µl) were isolated using the miRCURY™ RNA Isolation Kit-Biofluids (Exiqon, cat no-300112, Exiqon, Denmark) according to the manufacturer's instructions and quantified by RT-qPCR and analyzed as above. miR-103a (Exiqon) was used as internal control.

mRNA levels in the liver were measured by RT-qPCR using total RNA. cDNA synthesis were performed using Verso cDNA Synthesis kit (Thermo Scientific; USA) as per the manufacturer's instructions. RT-qPCR was performed using DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific; USA) and gene-specific primers from Sigma-Aldrich, India (Table S3) followed by melting curve analysis in an ABI VIIA7 (Applied Biosystem, Foster city, CA) cycler. 18S ribosomal RNA was used as invariant control. Results were analyzed by comparative ΔΔCt method.[16]

STATISTICAL ANALYSES

For identification of differentially expressed miRNAs test pValue (Unpaired Student's t-test with Benjamini Hocheberg FDR correction) threshold adjusted for false discovery rate of <0.05 was considered. miRNA target based pathway analysis done using miRPath was done with a pValue

threshold of <0.05 (Fischer's Exact Test) along with FDR 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 the significance difference 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 finally 263 patients were enrolled in this study (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. The whole genome miRNA expression profiling was done by microarray on RNA isolated from 80 subjects. Remainder 183 samples were stored for validation studies. The patients and healthy subjects were of either sex and between 35 to 55 years of age. The serum bilirubin and albumin levels in the healthy subjects and all the patients groups except the AVH group were in the normal range. The serum bilirubin, ALT and aspartate aminotransferase (AST) levels were much higher in the AVH group (Table 1).

ANALYSIS OF DIFFERENTIALLY EXPRESSED miRNAs AND THEIR CLUSTERS

The miRNA array data was analyzed by unsupervised Principal Components Analysis (PCA) as well as by hierarchical clustering to distinguish (similarity versus difference) between various patient groups.[17] Only healthy subjects and patients with 'advanced fibrosis' formed

independent clusters. Further, three of the five patient groups, viz., 'early fibrosis, 'immune tolerant' and 'no fibrosis' showed close association. Patients with 'early fibrosis' showed partial separation from the other disease groups while those with 'acute viral hepatitis' 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 different patient groups (Fig. 1B).

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 as compared to healthy controls (Table S4). In the AVH patients, 12 miRNAs were-up regulated and 2 miRNAs were down-regulated in comparison to IT group. Between 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 4 miRNAs were specifically down regulated. Further, distribution of differentially expressed miRNAs indicate a highly disease stage-specific up and down regulation of miRNAs, suggesting a very specific miRNA-mediated post-transcriptional gene regulation in each stage during disease progression (Fig. 2). Only 3 up-regulated miRNAs were common between IT versus healthy or AVH groups (Fig. 2A and lower panel). Interestingly, except for the IT group, fewer miRNAs were showed down-regulation in each disease group with virtually no overlapped expression (Fig. 2B).

BIOLOGICAL ANALYSIS OF DIFFERENTIALLY EXPRESSED miRNAS

The levels of differentially expressed miRNAs were analyzed by unsupervised hierarchical clustering using Pearson uncentered algorithm with average linkage rule. (Fig. 3). The differentially expressed miRNAs show a good separation between healthy and immune tolerant

patients (Fig. 3A) as well as a partial separation between acute viral hepatitis and immune tolerant (Fig. 3B). For example miR-3647-5p and 766-3p that were overexpressed in healthy group, were specifically shut down in IT group whereas miRNAs expressed at low levels healthy group (e.g., miR-20a-3p and miR-340-3p) were up-regulated in IT group (Fig. 3A). Similarly, miR-3613-3p and miR-129-2-3p showing low expression in AVH group, were up-regulated in 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 and 3D) suggesting multifactorial etiology of liver fibrosis. Furthermore, the heat maps are in agreement with the PCA and the hierarchical clustering which also showed no clear-cut separation among immune tolerant, no fibrosis, early fibrosis and AVH. Next using KEGG pathways, we performed enrichment analysis for differentially expressed miRNAs in hepatitis B patients. Significantly enriched KEGG pathways (p value <0.05) are shown in Fig. 4.

NETWORK OF miRNAS AND THEIR TARGET GENES INVOLVED IN HEPATITIS B PATHOPHYSIOLOGY

Next, the differentially expressed miRNAs were subjected to target based pathway enrichment analysis to identify key nodes and edges in each interpretation groups 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

As shown in Fig. 5A, the levels of miR-199a-5p, miR-221-3p and let-7a-5p were elevated in the IT patients that do not show any discernible biochemical change or hepatocellular injury but show active viral replication and persistence possibly by targeting host genes important for

innate immune suppression and HBV replication. Importantly, RT-qPCR 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 as compared to healthy controls whereas their cognate target genes, viz., *DDX3X*, *STAT2* and *TBK1* were down-regulated (Fig. 6A).

miRNAs identified with acute viral hepatitis

Fig. 5B, shows up-regulation of miR-125b-5p and miR-3613-3p and down-regulation of miR-940 in AVH group. Both miR-125b-5p and miR-3613-3p target STAT3 expression required for sustaining cell proliferation, differentiation and survival. Again, the array data was confirmed by RT-qPCR 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 low for being 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 HSCs that produce various types of extracellular matrix in response to TGF-beta and platelet-derived growth factor.[18] However, the miRNA regulation of early fibrosis is poorly understood. Our network analysis 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) which could be confirmed by RT-qPCR (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 extracellular matrix proteins cause gradual substitution of liver parenchyma and distortion of hepatic architecture called advanced liver fibrosis. Our network analysis identified 7 miRNAs associated with advanced fibrosis. Of these, five miRNAs, viz., miR-1, miR-10b-5p, miR-96-5p, miR-133b and miR-671-5p were up-regulated which can target the expression of some key transcription factors including ATF2, E2F3 and CREB3L2 (Fig. 5D). However, miR-20b-5p and miR-455-3p that are known to target cell cycle inhibitor $p21^{Cip1}$ gene were down-regulated in these patients. Further, RT-qPCR 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 liver could also be released in blood circulation via extracellular vesicles and exosomes, we also measured the levels of all 17 differentially expressed miRNAs by RT-qPCR in patient sera. The RT-qPCR 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) which otherwise is 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 was 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 7 miRNAs, viz, 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) albeit 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 in different stages of the diseases.[8,12] We have analyzed differential expression of 17 miRNAs in the liver biopsy samples of patients with different stages of HBV-associated liver diseases. 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 is a known regulator of some key genes required for normal liver physiology.[19] miR-199a can down-regulation of DEAD-box RNA helicase 3 and facilitate HBV replication in hepatocytes leading to viral persistence.[20,21]) miR-199a-3p regulates the expression of mammalian target of rapamycin and hepatocyte growth factor receptor c-Met in HCC cells.[22] 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.[22]

miR-221-3p reportedly targets kinase TBK1 which is important for interferon production and virus clearance.[22] Expectantly, 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.[23]

Let7a-5p, a tumor suppressor miRNA, was found to be up-regulated in the immunetolerant subjects. However, in cell culture, viral HBx down-regulates let-7a expression and supports cell proliferation by up-regulating STAT3.[24] The reasons for elevated levels of Let-7a in the IT

patients is not clear. However, elevated Let-7a-5p levels is known to affect innate immune response via transcription factor STAT2 and promote viral persistence.[25] Further, elevated let-7a levels positively correlate with HBV replication in HCC tissues.[26] These data suggest that elevated levels of miR-199a-5p, miR-221-3p and let-7a-5p apparently contribute towards suppression of 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 miR-221-3p level remained up (Fig. 7A). Thus, serum miR-221-3p could be considered as a biomarker for identification of the HBV infected IT patients.

miRNAs involved in acute viral hepatitis 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 TGF-β1 to activate HSCs 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 as a therapeutic target in liver cancer, the therapeutic use of miR-125b-5b and miR-3613-3p needs further investigation.[31] However, the serum levels of miR-125b-5p as well as miR-3613-3p were low in the AVH patients (Fig. 7B) suggesting their limited application as biomarker. Interestingly, CHB patients show elevated plasma levels of miR-125-5p perhaps to suppress viremia.[32]

miR-940 is a highly expressed miRNA in stomach tissue as compared to other tissues such as liver, breast, thyroid, and lung.[33] Interestingly, in our study, the miR-940 levels were down-

regulated in the liver biopsies and sera of AVH patients (Fig. 6B and 7B). miR-940 is reported to inhibit migratory and invasive potential of cells.[34,35] miR-940 is known to engage several crucial cellular pathways such as transcription factor SMAD4, TGF-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 miRNAs in gastric cancer, pancreatic ductal adenocarcinoma and HCC.[37,38] It shows progressively lower expression in early to advanced stage of gastric cancer and thus has the 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 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 (Fig. 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 phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) and Growth Factor Receptor Bound Protein 10 (GRB10) expression, miR-1224-3p will down-regulate AKT2 (V-Akt Murine Thymoma Viral Oncogene Homolog 2) and ELK1 (ETS domain-containing protein) while miR-1227-3p will target Phosphatase and tensin homolog (PTEN) and Heparan Sulfate Proteoglycan 2 (HSPG2). A recent report suggests that HSCs can be readily activated in the liver of PTEN null mouse as compared to control mouse liver. However, deletion of AKT2, the downstream target of PTEN signal, blocks NASH development, and alleviates fibrosis.[39]

Further, miR-1224-3p target Elk1 is considered as an important regulator of liver regeneration.[40] However, the biological relevance and diagnostic potential of ~~both~~ 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 early stage fibrotic patients with HBV and HCV infection, the 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 albeit its serum levels were found elevated. miR-499a-5p is reported to facilitate the expression of kinase inhibitors CDKN1 (Cyclin-dependent kinase inhibitor 1 or $p21^{Cip1}$) and IKBKB (Inhibitor of nuclear factor kappa-B kinase subunit beta or IKK-beta). While hepatic activation of IKK/NF-kB 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] IKKbeta 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 cell 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 ECM proteins (by fibrolysis), with the consequent onset of fibrotic scarring. In the HBV related cirrhotic population, we identified seven unique miRNAs. Of these, five miRNAs, viz., 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 viz., ATF2, E2F3 and CREB3L2 (Fig. 5D and 6D). Importantly, the expression of ATF2 target genes is frequently down-regulated in human cancers suggesting their involvement during tumor development.[44] Increased expression of miR-1 is significantly associated with chronic HBV infection, perhaps due to induction of impaired immune responses in these patients,[45] while its reduced serum levels have been proposed as a good prognostic marker for HCC.[46] It has also been suggested that elevated levels of miR-10b could serve as a noninvasive biomarker for predicting liver metastasis[47] while serum miR-96 could be a good biomarker for HCC patients with chronic HBV infection.[48] 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 cell cycle regulatory $p21^{Cip1}$ gene which may be involved in the regulation of cellular senescence in the diseased liver. Importantly, the patient sera in this patient group carried elevated levels of most microRNAs except miR-455-3p which need to be validated as biomarker (Fig. 7D).

To summarize, this novel 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.[49] 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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Conflict-of-interest statement: The authors have no conflict of interest to report

Supporting Information:

Fig. S1. Consort diagram of the study to show the flow of participants from assessment for eligibility to enrollment to whole genome expression analysis and q-PCR validation.

Table S1. Microarray data of differentially expressed miRNA in liver biopsies

Table S2. List of oligonucleotide primers used for RT-qPCR for miRNAs

Table S3. List of oligonucleotide primers used for RT-qPCR for target genes

Table S4. Number of differentially expressed miRNAs in each interpretation group

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Figure Legends

FIG. 1. Unsupervised Principal Component Analysis (PCA) and Hierarchical Clustering of different HBV patient groups profiled for miRNA expression. PCA (A) and hierarchical cluster analysis (B) were performed to show all subjects included in this study after color-coding according to the patient group. The patient groups were based on their whole genome miRNA expression profiles by ways of distance matrix and co-variance. All patient groups except for 'early fibrosis' appeared to form independent clusters. The proximity among various groups seems to reflect a low 'between-group' variance with some overlaps. The tree scale is shown at the bottom of panel B. Euclidean distance was used as a measure between points.

FIG. 2. Venn Diagram Representation of up- and down-regulated miRNAs in different comparison groups of hepatitis B patients. Distribution of differentially expressed specific and overlapping miRNAs in four comparison groups of hepatitis B patients and healthy control (Yellow = immune tolerant vs healthy; Green = acute viral hepatitis vs immune tolerant; Blue = early fibrosis vs no fibrosis; Red = advanced fibrosis vs. early fibrosis). The shadows of corresponding colors represent overlapping miRNAs. The numbers and their percentage indicate disease and stage-specific up- or down regulation of miRNAs suggesting a strong post transcriptional gene regulation involvement in disease progression.

FIG. 3. Unsupervised clustering of most differentially expressed miRNAs compared between different HBV patient groups and healthy control. Pearson uncentered algorithm with average linkage rule based unsupervised hierarchical clustering of differentially expressed transcripts showing distinct patterns of up and down regulated miRNAs. These miRNAs are specific to disease stage and reproducibility in their absolute expression levels within the cohort

profiled. The columns represent the analyzed samples, while the rows represent the 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.

FIG. 4. Bubble plot representation of the signaling and biochemical pathways affected by differentially expressed miRNAs in the liver of hepatitis B patients. This plot reveals regulation of selective signaling pathways by different cluster of miRNAs under various clinico-pathological conditions associated with HBV. The Y axis shows term enrichment of miRNAs involved in targeting various pathways (shown as colored modules) while the X axis shows enrichment significance (p Value) of the signaling and biochemical pathways. Numbers in parenthesis refer to the pathway identifier in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

FIG. 5. Interactive network of miRNA and target genes involved in hepatitis B pathophysiology. The miRNA gene regulatory network consisted of 17 miRNAs and 18 target gene interactions where diamonds represent miRNAs and circular nodes target genes. This network clearly shows disease stage-specific gene regulation by miRNAs involved in Hepatitis-B disease progression. Nodes (genes and miRNAs) are colored as per their expected expression levels with reference to the disease stage (red as induction / over expression and green as repression / down regulation). The edges (connections) are shaped as per their role in gene regulation where T ends (⊣) indicate gene inhibition while arrows (→) indicates gene stimulation.

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 were analyzed by

RNA-qPCR in the RNA samples isolated from the liver biopsies of different patient groups and healthy control (15 samples per group) to confirm the microarray data. The 18S ribosomal RNA was used as internal control for target genes whereas and U6 RNA was used as internal control for miRNAs. The PCR primer sequences for miRNAs and their target genes are given in Table S2 and S3 respectively. Bar charts represents qRT PCR results of miRNAs in Immune tolerant group versus healthy control (A), AVH-B group versus Immune tolerant group (B), early fibrosis group compared to no fibrosis group (C) and in advanced fibrosis group versus early fibrosis group (D). All experiments were performed in triplicate. Data are shown as mean ± SD. ** and * represent significance with p values <0.01 and <0.05 respectively.

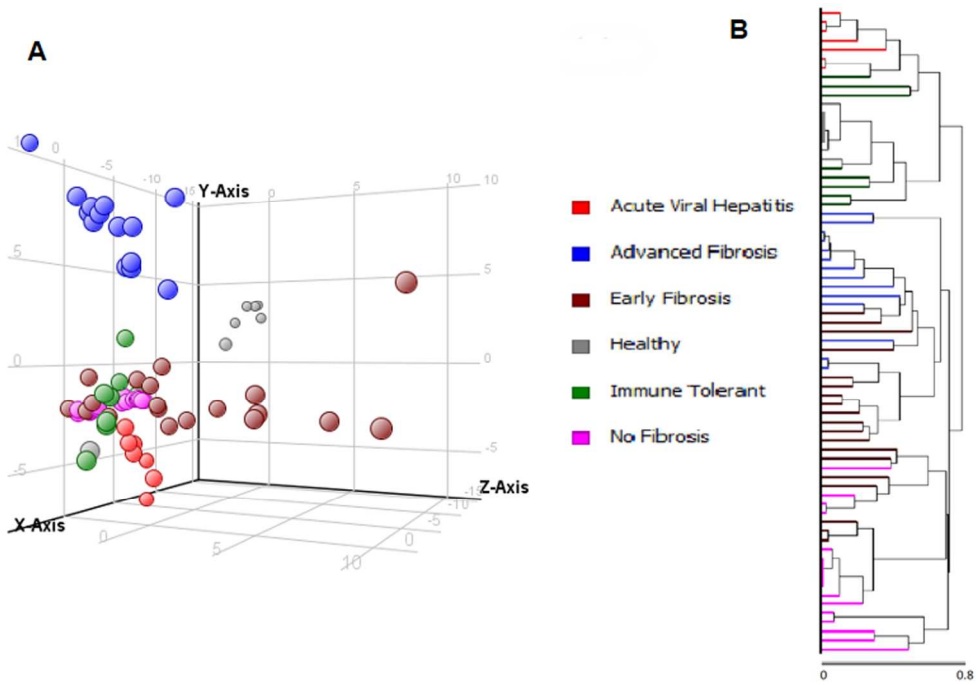
FIG. 7. Validation of circulating miRNA in the sera of hepatitis B patients. The levels of 17 differentially expressed miRNAs observed in the liver, were also determined by RT-qPCR in the sera of 5 patients in each patient group. miR-103a (Exiqon) was used as control. The primer sequences are given in Table S2. Bar charts represents qRT PCR results of miRNAs in Immune tolerant group versus healthy control (A), AVH-B group versus Immune tolerant group (B), early fibrosis group compared to no fibrosis group (C) and in advanced fibrosis group versus early fibrosis group (D). All experiments were performed in triplicate. Data are shown as mean ± SD. ** and * represent significance with p values <0.01 and <0.05 respectively.

Table 1. The demographic and clinical characteristics of the enrolled patients and healthy subjects.

[Unless otherwise indicated, all values are shown as median along with (range)]

Parameters	Healthy (N=23)	AVH-B (N=30)	Immune Tolerant (N=30)	No Fibrosis (N=60)	Early Fibrosis (N=60)	Advanced Fibrosis (N=60)	p Value*
Age (Yrs)	41.00 (37.00-59.00)	44.50 (33-62)	39.0 (20.00-63.00)	35.50 (20.00-63.00)	34.00 (20.00-56.00)	45.50 (34.00-66.00)	0.170
Male gender (%)	65.7	63.5	82.5	83.5	84.5	81.2	0.06
Total Bilirubin (mg/dL)	0.80 (0.60-1.00)	13.90 (5.0-20.78)	0.80 (0.70-1.30)	0.80 (0.66-1.30)	0.80 (0.40-2.20)	0.90 (0.49-2.93)	0.00
Serum Bilirubin Direct (mg/dL)	0.08 (0.05-0.10)	8.65 (3.5-11.0)	0.10 (0.02-0.60)	0.10 (0.02-0.60)	0.10 (0.00-0.30)	0.10 (0.05-0.30)	0.00
Serum Bilirubin Indirect (mg/dL)	0.75 (0.50-0.95)	5.25 (1.5-9.91)	0.70 (0.51-0.74)	0.70 (0.51-1.01)	0.70 (0.35-1.90)	0.70 (0.44-2.65)	0.00
Total Protein Biuret (g/dL)	7.70 (7.10-8.20)	7.3 (6.3-8.00)	7.15 (6.30-7.50)	7.30 (6.30-9.10)	7.30 (6.10-8.20)	7.90 (5.80-8.40)	0.20
Albumin (g/dL)	4.50 (4.00-4.80)	3.35 (1.7—3.80)	3.93 (3.20-4.50)	4.10 (3.20-4.50)	4.10 (2.90-4.80)	4.05 (2.90-4.70)	0.00
Globulin (g/dL),	3.20 (3.00-3.70)	4.15 (3.20-4.80)	3.10 (2.40-3.70)	3.20 (2.40-4.90)	3.30 (2.60-3.70)	3.60 (2.90-4.60)	0.00
ALT (IU/L),	32 (23-37)	1118 (450-2378)	26.00 (11.00-37.00)	35 (11-120)	71 (20-220)	38 (21-72)	0.00
AST (IU/L),	20 (18-32)	594.50 (420-2009)	24.95 (18-36)	26 (18-39)	50 (23-104)	33 (18-54)	0.00
SAP (IU/L)	74 (63-79)	134.0 (94.0-325.0)	54.00 (35.00-117.00)	74 (35-117)	83 (50-215)	80 (54-169)	0.00
GGT (IU/L)	16 (10-36)	69.0 (47-277)	16 (8-32)	16.50 (8.00-32.00)	20 (8-57)	29 (14-101)	0.00
AFP (ng/mL)	2.0 (1.93-2.08)	6.48 (1.97-77.53)	2.84 (1.23-8.66)	3.24 (1.23-8.66)	4.85 (1.77-8.52)	4.43 (1.82-11.70)	0.07
INR	1.0 (0.90-1.10)	1.15 (1.00-1.29)	1.0 (0.90-1.20)	1.00 (0.90-1.20)	1.00 (0.90-1.20)	1.10 (1.00-1.64)	0.09
Log10 [HBsAg] (IU/mL)		4.24 (3.33-5.12)	3.59 (2.70-4.00)	3.68 (2.70-5.25)	4.37 (2.86-5.40)	3.87 (2.09-5.29)	0.40
Log10 [HBV DNA] (IU/mL)		5.81 (1.49-7.28)	7.01 (6.54-7.14)	4.09 (2.49-8.04)	4.32 (0.78-8.04)	4.54 (0.78-8.04)	0.65

*, The nonparametric Kruskal-Wallis test was applied to assess the significance difference at $p < 0.05$). ALT, alanine aminotransferase; AST, aspartate aminotransferase; SAP, serum amyloid P component; GGT, gamma-glutamyl transferase; AFP, alpha-fetoprotein; INR, international normalized ratio.



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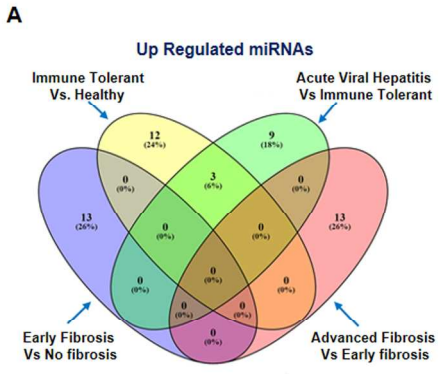

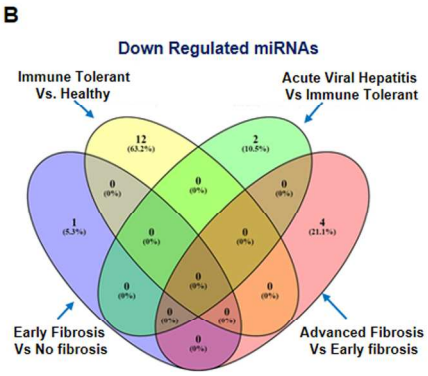

Group Comparison	Up-regulated miRNAs	
Immune tolerant vs Healthy	hsa-let-7a-5p ; hsa-miR-125b-2-3p ; hsa-miR-16-2-3p ; hsa-miR-199a-5p ; hsa-miR-20a-3p ; hsa-miR-221-3p ; hsa-miR-24-1-5p ; hsa-miR-301a-3p ; hsa-miR-337-5p ; hsa-miR-338-3p ; hsa-miR-33a-5p ; hsa-miR-532-3p	Common miRNAs
Acute Viral Hepatitis vs Immune Tolerant	hsa-miR-101-3p; hsa-miR-125b-5p ; hsa-miR-129-1-3p; hsa-miR-129-2-3p ; hsa-miR-193b-5p ; hsa-miR-30c-5p ; hsa-miR-30e-3p ; hsa-miR-3613-3p ; hsa-miR-365a-3p	hsa-miR-340*; hsa-miR-500a; hsa-miR-4324
Early Fibrosis vs No fibrosis	hsa-miR-1224-3p ; hsa-miR-1227-3p ; hsa-miR-224-3p ; hsa-miR-34b-3p ; hsa-miR-3647-5p ; hsa-miR-3681-3p ; hsa-miR-3685 ; hsa-miR-382-5p ; hsa-miR-411-3p ; hsa-miR-647 ; hsa-miR-7-2-3p ; hsv2-miR-H9-3p ; kshv-miR-K12-12*	
Advanced Fibrosis vs Early fibrosis	hcmv-miR-UL70-3p ; hsa-miR-1-3p ; hsa-miR-10b-5p ; hsa-miR-133b ; hsa-miR-145-5p ; hsa-miR-199a-3p ; hsa-miR-218-5p ; hsa-miR-23a-3p ; hsa-miR-551b-3p ; hsa-miR-663a ; hsa-miR-671-5p ; hsa-miR-96-5p ; hsv1-miR-H17	

Group comparison	Down-regulated miRNAs	
Immune tolerant vs Healthy	hsa-miR-1202 ; hsa-miR-1234-3p ; hsa-miR-1281 ; hsa-miR-1825 ; hsa-miR-2278 ; hsa-miR-3162-5p ; hsa-miR-3647-5p ; hsa-miR-574-3p ; hsa-miR-574-5p ; hsa-miR-766-3p ; hsv1-miR-H6-3p ; hsv2-miR-H5-3p	Common miRNAs
Acute Viral Hepatitis vs Immune Tolerant	hsa-miR-3665 ; hsa-miR-940	None
Early Fibrosis vs No fibrosis	hsa-miR-499a-5p	
Advanced Fibrosis vs Early fibrosis	hsa-miR-20b-5p ; hsa-miR-3675-3p ; hsa-miR-455-3p ; hsa-miR-505-3p	

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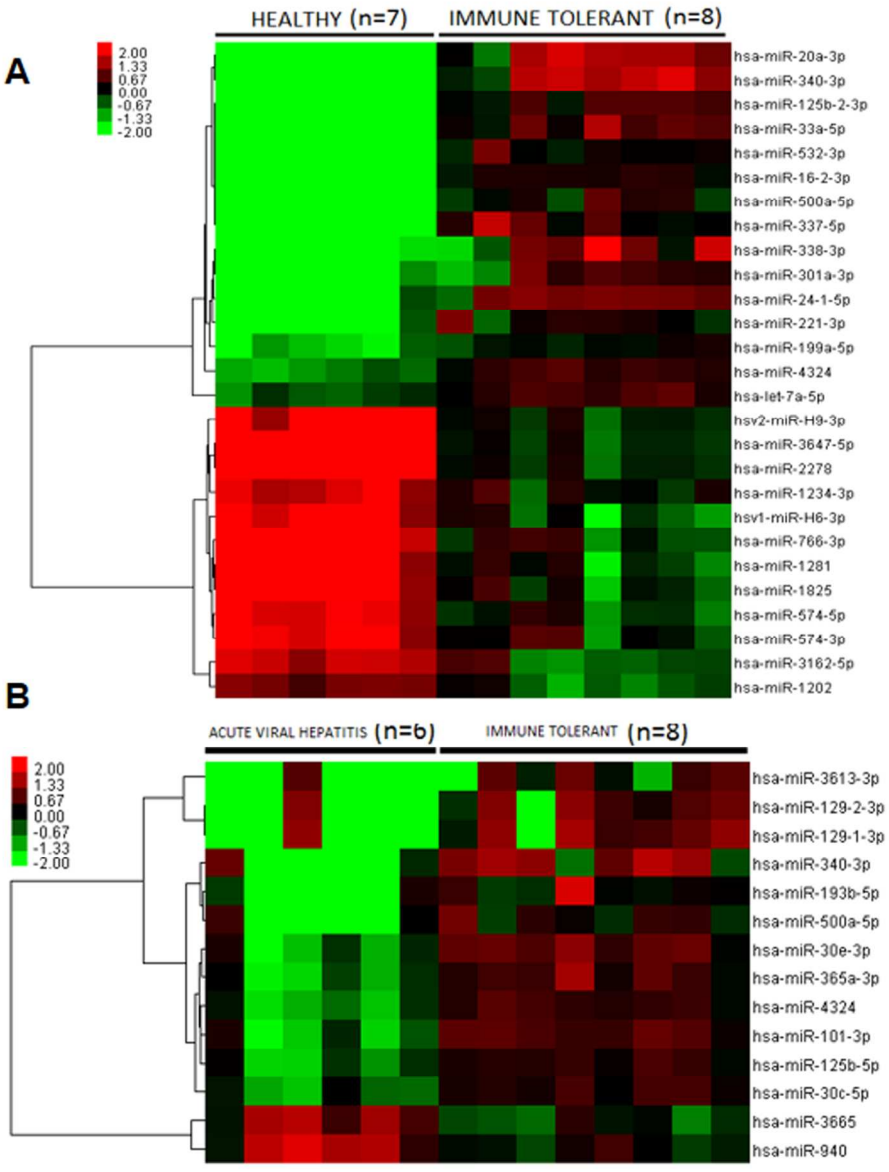

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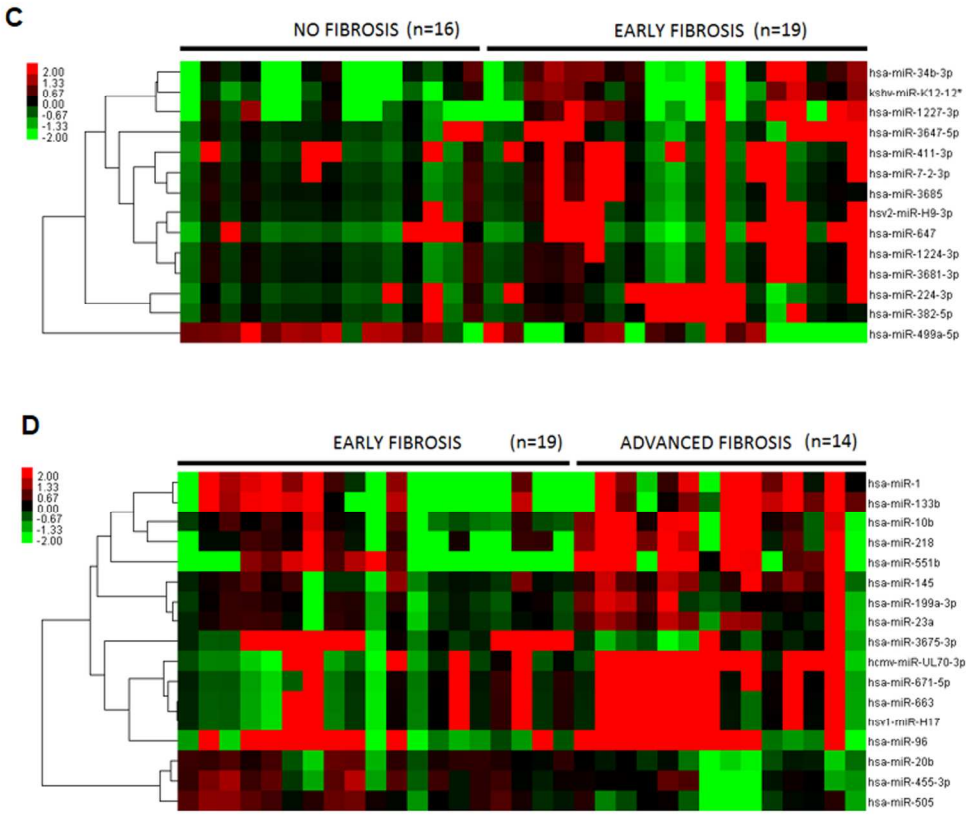

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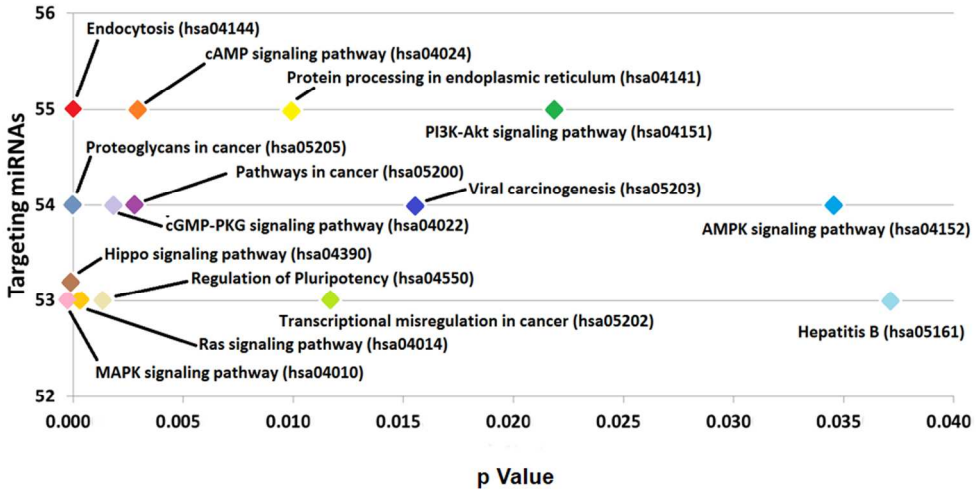

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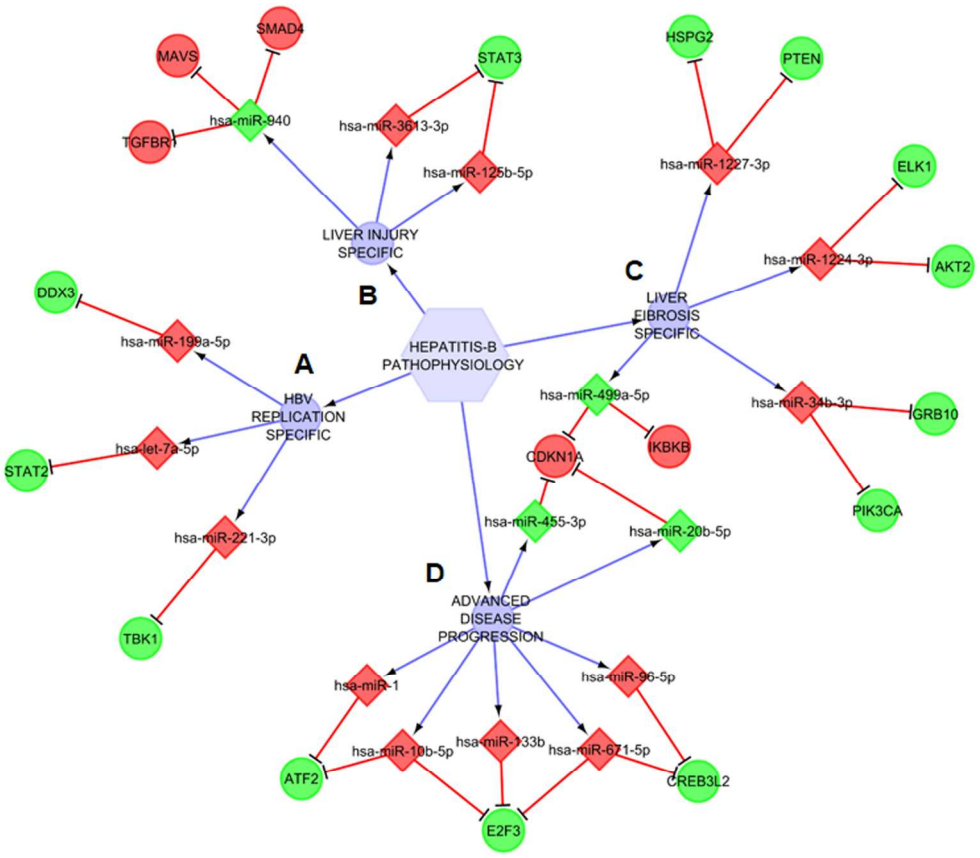

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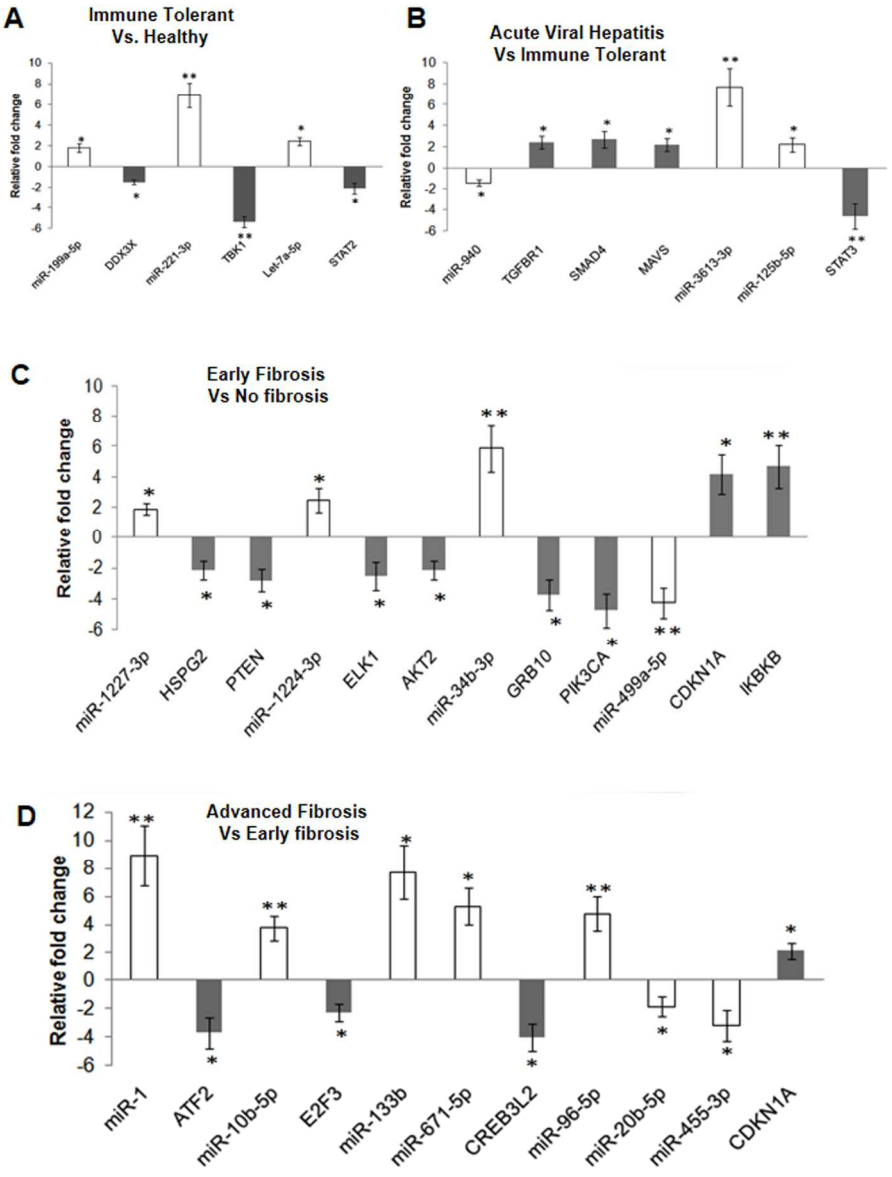

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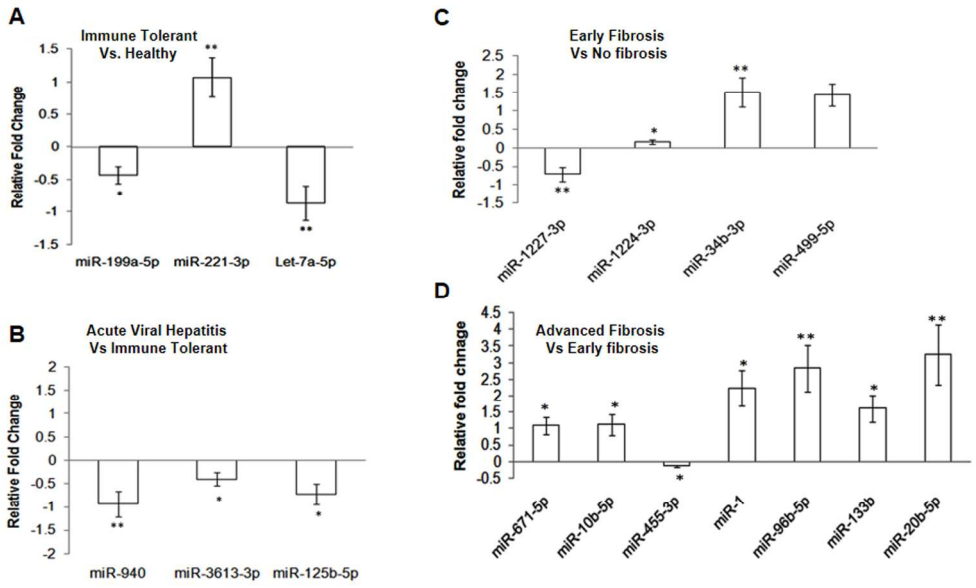

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