RESEARCH ARTICLE

Identification of miRNAs associated with dendritic cell dysfunction during acute and chronic hepatitis B virus infection

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Abstract

The uptake or expression of hepatitis B virus (HBV) proteins by dendritic cells (DCs) is considered important for disease outcome. Differential expression of microRNA (miRNA) may have a role in viral persistence and hepatocellular injury. The miRNA expression was investigated by microarray in DCs from different stages of HBV infection and liver disease namely, immune active (IA; $n = 20$); low replicative (LR; $n = 20$); HBeAg negative ($n = 20$); acute viral hepatitis (AVH, $n = 20$) and healthy controls ($n = 20$). miRNA levels were analyzed by unsupervised hierarchical clustering and principal component analyses and validated by quantitative polymerase Chain Reaction (qPCR). The miRNA-messenger RNA (mRNA) regulatory networks identified 19 miRNAs and 12 target gene interactions in major histocompatibility complex and other immune pathways. miR-2278, miR-615-3p, and miR-3681-3p were downregulated in the IA group compared to healthy control, miR-152-3p and miR-3613-3p in the LR group compared to IA group and miR-152-3p and miR-503-3p in HBe negative compared to LR group. However, miR-7-1-1-3p, miR-192-5p, miR-195-5p, and miR-32-5p in LR, miR-342-3p, and miR-940 in HBe negative, and miR-34a-5p, miR-130b-3p, miR-221-3p, miR-320a, miR-324-5p, and miR-484 in AVH were upregulated. Further, qPCR confirmed changes in miRNA levels and their target genes associated with antigen processing and presentation. Thus, a deregulated network of miRNAs-mRNAs in DCs seems responsible for an impaired immune response during HBV pathogenesis.

KEYWORDS

acute viral hepatitis, antigen presentation, chronic hepatitis B, HBe antigen, microarray, RT-qPCR

1 | INTRODUCTION

The hallmark of chronic hepatitis B (CHB) infection is the lack of a robust hepatitis B virus (HBV)-specific CD8⁺ and CD4⁺ T-cell response leading to the induction of virus-specific T and B cell responses. HBV can downregulate host immune responses by interfering with toll-like receptors (TLR) expression and signaling pathways or via inhibition of interferon response.¹ Besides, the expression of HBV proteins in/by dendritic cell (DC) can lead to impairment of their immune-stimulatory function, causing insufficient/ineffective priming of CD4⁺ T cells, possibly contributing to the persistence of viral hepatitis.² DCs do not support HBV replication but can take up HBV proteins which may affect DC activity.³ Binding and uptake of HBV virions by DCs may also be responsible for the impaired function of DC in HBV-infected patients. Functional alterations in DC or its precursors from chronic HBV patients also contribute to the impaired HBV-specific immune response.^{4,5} Patients with advanced fibrosis or cirrhosis show a decrease in DC numbers in peripheral blood of chronic HBV patients compared to healthy individuals.^{5,6} Not surprisingly, DCs from CHB patients are less efficient in inducing T-cell proliferation *in vitro* than those isolated from healthy individuals^{6,7} and show low expression of IFN- α and DC-specific intercellular adhesion molecule 3-grabbing non-integrin which may affect initial events of immune activation.⁸ However, some clinical studies have shown that DCs in chronic hepatitis B carriers are functionally intact.⁹ Thus, DCs appear to be a central player in initiating and controlling the magnitude and quality of adaptive immune responses.⁴ However, the molecular mechanisms associated with DC dysfunction in CHB patients remain poorly understood.

Aberrant expression of certain micro RNAs (miRNAs) can lead to pathological consequences in many liver pathologies including viral hepatitis, acute and chronic liver diseases, and hepatocellular carcinoma.^{10,11} Therefore, a detailed investigation on the expression of miRNAs in the DCs should provide an in-depth understanding of the molecular mechanisms associated with immune-compromised status in CHB patients and also useful in the management of liver diseases.^{12,13} The natural history of chronic HBV infection is a dynamic process that can progress gradually or rapidly consisting of four phases of the underlying liver disease, of variable duration and outcome. All phases have been pathogenetically linked to the level of HBV replication and the strength and targets of the host immune reactivity against the replicating HBV. In childhood infection, the first phase is called as "immune-tolerant phase" characterized by normal serum levels of alanine aminotransferase (ALT) and very high levels of HBV DNA with minimal fibrosis and inflammation in the liver. The majority of chronically infected individuals can develop "immune active (IA) phase" (second phase) characterized by elevated ALT and high HBV DNA levels and persistent immune-mediated necroinflammatory activity. The third phase is called "HBeAg-negative chronic infection" characterized by low-level HBV DNA, raised ALT with significant liver disease on liver biopsy. The fourth phase is called a "LR phase," characterized by low HBV DNA level, persistent normal ALT with no significant liver disease on liver

biopsy. Here, we have analyzed the aberrant expression of miRNA and their target genes in DCs from freshly separated PBMC of hepatitis B patients with following distinct phenotypes: (i) acute viral hepatitis, (ii) IA, (iii) LR, and (iv) HBeAg negative CHB individuals. Our study suggested the involvement of 19 miRNAs and 12 target genes for DC-related immune dysfunction in hepatitis B patients.

2 | METHODS

2.1 | Study cohort

HBV-infected patients visiting the Institute of Liver and Biliary Sciences, New Delhi were screened and enrolled in this study. The study protocol was approved by the Ethics Committee of ILBS. Informed consent was obtained from all patients and their detailed history recorded. The inclusion criteria for the four 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) IA: HBsAg positive patients for more than 6 months, HBeAg positive, anti-HBe negative, high DNA (>20,000 IU/ml), raised ALT and significant liver disease on liver biopsy (HAI >3, fibrosis score ≥ 2); (iii) LR: patients with HBsAg positive for >6 months, HBeAg negative, anti-HBe positive, DNA (<2,000 IU/ml), persistent normal ALT and no significant liver disease on liver biopsy (HAI ≤ 3 , fibrosis score <2); (iv) HBeAg negative CHB patients with HBsAg positive for >6 months, HBeAg negative, anti-HBe positive, DNA (>2000 IU/ml), raised ALT and significant liver disease on liver biopsy (HAI >3, fibrosis score ≥ 2); (v) healthy controls: age, sex and nutritional status matched asymptomatic healthy individuals with no history or clinical features of any liver disease and with normal levels of serum transaminases and negative for IgM anti-HAV, IgM anti-HEV, HBsAg, anti-HBe, IgG anti-HBc markers. Immune tolerant (Phase I) patients were not included in this study. All patients were treatment naïve at the time of sampling.

Exclusion criteria included co-infection with hepatitis C, hepatitis D, or HIV; decompensated liver disease; chronic hepatitis B with Child-Pugh B and C; liver disease of unknown etiologies; serum creatinine more than 1.5 times upper limit of normal; hemoglobin <10 g/dl; platelet and WBC counts were <70,000 and <3000 per cubic millimeter respectively; no serious concurrent medical illnesses like malignancy, severe cardiopulmonary disease, uncontrolled diabetes mellitus, alcohol consumption more than 20 g/day.

2.2 | Isolation of dendritic cells and RNA

DCs were isolated from freshly separated PBMCs using a blood DC isolation kit (Miltenyi Biotec, Cat No: 130-091-379) as per the manufacturer protocol. The purity of the DC population was ascertained by flow cytometry after staining with fluorochrome-conjugated antibodies against CD14, CD19, BDCA-1, BDCA-2, and BDCA-3. DCs with

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

Parameters	AVH-B (N = 20)	HBsAgNeg CHB (N = 20)	Low Replicative (N = 20)	Immune Active (N = 20)	Healthy (N = 20)	p Value*
Age (years)	47.50 (29.00–69.00)	34.00 (22.00–66.00)	36.50 (23.00–68.00)	35.00 (21.00–53.00)	38.00 (29.00–59.00)	.14
Male gender (%)	69.50	82.5	78.00	71.25	64.3	.06
Total bilirubin (mg/dl)	19.30 (12.40–20.39)	0.80 (0.46–1.80)	0.875 (0.40–1.90)	0.90 (0.50–1.39)	0.80 (0.6–1.0)	.001
Serum bilirubin direct (mg/dl)	11.03 (10.21–14.40)	0.10 (0.04–0.50)	0.10 (0.01–0.30)	0.15 (0.10–0.30)	0.08 (0.005–0.10)	<.001
Serum bilirubin indirect (mg/dl)	7.89 (0.50–9.32)	0.70 (0.33–1.6)	0.75 (0.32–1.70)	0.70 (0.40–1.24)	0.75 (0.50–0.95)	.063
Total protein biuret (g/dl)	7.25 (6.80–8.40)	7.45 (5.90–9.30)	7.40 (6.30–8.70)	7.50 (6.70–9.00)	7.7 (7.10–8.20)	.70
Albumin (g/dl)	2.85 (1.70–3.80)	4.25 (2.10–4.80)	4.40 (2.70–4.90)	4.15 (3.70–4.50)	4.50 (4.00–4.80)	.002
Globulin (g/dl)	4.30 (3.70–6.70)	3.30 (2.50–4.50)	3.20 (2.40–4.60)	3.30 (2.80–5.30)	3.40 (3.00–3.70)	.016
ALT (IU/L)	527.50 (440.50–2251.00)	46.50 (42.00–508.00)	26.00 (14.00–40.00)	70.00 (44.00–181.00)	32.00 (23.00–39.00)	<.001
AST (IU/L)	470.50 (410.50–1013.00)	43.00 (42.00–382.00)	24.50 (18.00–34.00)	58.00 (45.00–128.00)	27.00 (18.00–35.00)	<.001
SAP (IU/L)	113.50 (68.00–149.00)	83.50 (50.00–268.00)	65.00 (53.00–107.00)	78.50 (54.00–106.00)	74.00 (63.00–79.00)	.062
GGT (IU/L)	43.50 (16.00–64.00)	21.50 (8.00–605.00)	14.50 (9.00–55.00)	22.00 (13.00–79.00)	16.00 (10.00–36.00)	.022
AFP (ng/ml)	7.05 (2.74–22.26)	4.21 (1.68–18.16)	4.04 (1.25–8.28)	3.87 (1.20–12.06)	2.00 (1.93–2.08)	.134
INR	1.32 (1.20–3.05)	1.00 (0.90–1.20)	1.00 (0.95–1.20)	1.00 (0.9–1.10)	1.00 (0.90–1.10)	.016
Log10 [HBsAg] (IU/ml)	2.87 (1.70–4.93)	3.84 (2.16–5.10)	3.32 (1.54–4.75)	3.99 (2.23–4.77)		.093
Log10 [HBV DNA] (IU/mL)	3.66 (3.00–5.93)	3.80 (3.30–8.04)	2.56 (0.78–3.28)	7.18 (4.30–8.04)		<.001
HAI		4.00 (4.00–5.00)	2.00 (1.00–3.00)	4.00 (4.00–6.00)		.003
Fibrosis		2.00 (2.00–3.00)	0.00 (0.00–1.00)	2.00 (2.00–3.00)		.01

Note: (Unless otherwise indicated, all values are shown as median [minimum–maximum]).

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; HAI, histological activity index; INR, international normalized ratio; SAP, serum amyloid P component.

*p < .05 was considered significant.

>90% purity was used for isolating total RNA and miRNA as described previously.¹³

2.3 | Analysis of miRNA expression

The expression of miRNA was determined using Agilent's human miRNA microarray version V16 as described earlier.¹³ Unsupervised hierarchical clustering of differentially expressed genes between patient groups were done using Pearson uncentered algorithm with average linkage rule. The pathway enrichment analysis of miRNAs was done using DIANA-miRPath v3.0 while the miRNA:mRNA gene regulatory network was visualized using CytoScape V 2.8.2.¹³

2.4 | Quantitation of miRNA and target gene expression

The levels of miRNAs and their target mRNAs isolated from DCs were measured by real time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) as described previously.¹³ The details of miRNA and gene primers can be found in Tables S1 and S2 respectively. The results were analyzed by the $\Delta\Delta C_t$ method.¹⁴

2.5 | Statistical analysis

For identification of differentially expressed miRNAs the test *p* value (Unpaired Student's *t*-test with Benjamini Hochberg false discovery rate [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 *p* value 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 comparison of continuous data was carried out using the Kruskal-Wallis test followed by posthoc comparison by probability adjustment. *p* Value <0.05 considered significant.

3 | RESULTS

3.1 | Clinical characteristics of the enrolled patients with HBV infection

A total of 140 patients were assessed for eligibility criteria and finally 100 patients were enrolled in this study (Figure S1). The demographic and clinical characteristics of the enrolled HBV patients and healthy subjects are given in Table 1. Of these, 40 patients were excluded based on not meeting the inclusion criteria, declined to participate, and set exclusion criteria for the poor quality of RNA

isolated from DCs. The whole-genome miRNA expression profiling was done by microarray on RNA samples from 50 subjects. Remainder 50 samples were stored for validation studies. The serum bilirubin and albumin levels in the healthy subjects and all the patients groups except the AVH group were within the normal range. Posthoc comparison revealed that in the AVH group serum bilirubin, ALT, aspartate aminotransferase (AST) and albumin levels was statically significant compare to all other groups (Table 1).

3.2 | Analysis of differentially expressed miRNAs and their clusters in DCs

The miRNA array data were analyzed by principal components analysis (PCA) as well as by unsupervised hierarchical clustering to distinguish (similarity vs. difference) between various patient groups.¹⁵ Each patient group formed distinct clusters suggesting a high degree of reproducibility within replicate samples (Figure 1A). The hierarchical clustering analysis also showed distinct miRNA expression patterns as evident from greater branch length between healthy control, AVH, and chronic hepatitis B patients. Further, among chronic HBV patients, specific clustering was observed for HBeAg negative, IA and LR groups (Figure 1B).

Next, we analyzed the distribution of differentially expressed miRNAs for specificity and commonality in various patient groups and healthy subjects (Table S3). In immune active group compared to healthy controls, just nine miRNAs were downregulated (Table S4A). However, in low replicative patients, as compared to the immune active group, 44 miRNAs were upregulated and 4 miRNAs were downregulated (Table S4B). Between HBeAg negative and low replicative groups, 12 miRNAs were upregulated and 8 miRNA were downregulated (Table S4C). Importantly, in the AVH group compared to an immune active group, 58 miRNAs were upregulated and 27 miRNAs were specifically downregulated (Table S4D). The Venn diagram representation of miRNA expression also indicated group-specific up- or downregulation of miRNAs suggesting their close association with disease progression (Figure 2). Among upregulated miRNAs, 19 miRNAs were specific for low replicative versus immune active whereas 25 miRNAs were common between low replicative and AVH groups versus immune active group. In HBeAg negative compared to an immune active group, 11 miRNAs were unique while only 1 miRNA (miR-575) was shared between AVH versus immune active group which otherwise exhibited 32 specific miRNAs (Figure 2A). Among, downregulated miRNAs, 8 miRNAs were specific for Immune active compared to healthy groups whereas only 1 miRNA (miR-3907) was shared between HBeAg negative and low replicative groups which otherwise showed downregulation of seven specific miRNAs (Figure 2B). Only two miRNAs (miR-149 and miR-3613-3p) were unique in low replicative versus immune active comparison whereas the other two miRNAs (viz., miR-647 and miR-1296) were shared with AVH versus immune active groups. The AVH group as compared to immune active patients otherwise showed downregulation of 25 specific miRNAs (Figure 2B).

FIGURE 1 Unsupervised principle component analysis (PCA) and hierarchical clustering of various HBV patient groups based on miRNA expressed in dendritic cells. PCA (A) and hierarchical cluster analysis (B) were performed to show all subjects after color-coding each patient group based on miRNA expression profiles by ways of the distance matrix and covariance. The four patient groups (immune active, low replicative, HBe antigen negative, and AVH) clustered nicely and segregated from one another like healthy controls suggesting a sufficient “between-group” than “within group” variance. AVH, acute viral hepatitis; miRNA, micro RNAs

3.3 | Status of differentially expressed miRNAs in DCs

The levels of differentially expressed miRNAs were analyzed by unsupervised hierarchical clustering. Each patient group and healthy control showed specific miRNA expression patterns as revealed by unique heat maps (Figure 3). For example, miR-454, miR-615-3p, miR-605, miR2278, and miR-3681-3p were high in the healthy group but low in immune active patients whereas miR-3907, miR-500a*, miR-4313, and miR-4323 were downregulated in immune active patients (Figure 3A). Likewise, nearly 25 miRNAs including miR-500a*, miR-125b, miR-3907 were down in the immune active group, showed relatively elevated profiles in the low replicative groups of patients (Figure 3B). The levels of ~22 miRNAs including miR-152, miR-224, miR-22*, miR-210 were up in the low-replicative group (Figure 3B). Similarly, miR-191*, miR-1228, and miR-1238 was high in the HBe negative patients compared to the low replicative group whereas miR-152, miR-339-5p, and miR-339-3p were higher in the low replicative group (Figure 3C). In the AVH group as compared to the immune active group, miR-127-3p and miR-339-5p levels were elevated whereas miR-660 was down. Interestingly, levels of miR-30a, miR-7, miR-10a, miR-181c, miR-223*, and so forth were downregulated in the immune active patients (Figure 3D).

The differentially expressed DC miRNAs were predicted to engage multiple cellular and signaling networks essential for antigen processing and presentation, MHC I signaling, type I interferon signaling, TLR signaling, T cell receptor signaling, MAP kinase, and PI3K-Akt signaling and apoptosis and so forth. (Figure 4).

3.4 | Pathway enrichment analysis for differentially expressed miRNAs in DCs

Gene pathway enrichment for differentially expressed miRNAs was determined using a web-based pathway analysis program “Kyoto Encyclopedia of Genes and Genomes” (KEGG; <https://www.genome.jp/kegg/>). There was significant enrichment of specific pathways (p value < .05) which could impact primary immunodeficiency, antigen processing and presentation, T cell receptor signaling and protein folding, and so forth. as evident from up- (red) or downregulation (green) of selective pathways/molecules (Figure S2). In the immune active versus healthy group, some key genes involved in the antigen processing and presentation pathway were upregulated for both the MHC-I and MHC-II pathways.¹⁶ Elevated levels of calnexin and calreticulin, and chaperones HSP70 and HSP90 in MHC-I pathway could stimulate CD4 and CD8 T

FIGURE 2 Venn diagram representation of differentially expressed miRNA in dendritic cells isolated from various HBV patient groups and healthy control. Distribution of up- (A) and downregulated (B) specific and overlapping miRNAs in four comparison groups of hepatitis B patients and healthy control (Yellow = immune active vs. healthy; Green = low replicative vs. immune active; Blue = acute viral hepatitis vs. immune active; Red = HBe negative vs. immune active). The shadows of corresponding colors represent overlapping miRNAs. The numbers and their percentage indicate disease and stage-specific up- or downregulation of miRNAs in DCs during disease progression. Specific and overlapping miRNAs are shown below in boxes. HBV, hepatitis B virus; miRNA, micro RNAs

cell and NK cell functions (Figure S2A). The MHC-I pathway seemed to be downregulated in low replicative compared to immune active group resulting in low levels of calnexin, HSP70, HSP90, and proteasomal activator subunit PA28 whereas the MHC-II pathway remained unaffected (Figure S2B). In the antigen versus low replicative group, the MHC-I pathway may be activated despite low expression of calreticulin and HSP70 impacting CD8 T cell and NK cell functioning (Figure S2C). Further, in the AVH group as compared to immune active subjects, both MHC-I and MHC-II pathways should be downregulated (Figure S2D).

3.5 | Network of miRNAs and their target genes and their confirmation by RT-qPCR

The target-based pathway enrichment analysis of DC miRNAs showed a network of 19 miRNAs and 12 target gene interactions with multiple nodes each with interdependent gene regulation by miRNAs in MHC-I and MHC-II pathways to affect CD4 and CD8 T cell and NK cell functioning during hepatitis B-related disease progression (Figure S3E and Table S5).

There was specific downregulation miR-2278, miR-615-3p, and miR-3681-3p in the DCs of immune active group versus healthy control. Low levels of miR-615-3p and miR-3681-3p in DCs would result in elevated expression of calnexin (CANX) gene (Figure S3A) which functions as a chaperone for MHC class I α -chain.¹⁷ Further, downregulation of miR-615-3p, would lead to elevated expression of HSP70 (HSPA8 and HSPA1B) and HSP90 (HSP90AB1) genes which play important immunoregulatory functions, required for maturation of dendritic cells and induction of immune responses.¹⁸ As expected, the RT-qPCR analysis confirmed the microarray data and revealed decrease levels of miR-2278, miR-615-3p, and miR-3681-3p in this interpretation and elevated levels of their seven target genes, namely, CANX, CALR, HSPA1B, HSPA8, HSP90AB1, HLA-A and HLA-B (Figure 5A).

In low replicative compared to an immune active group, four miRNAs viz, miR-7-1-3p, miR-192-5p, and miR-195-5p and miR-32-5p were upregulated whereas miR-152-3p and miR-3613-3p were downregulated. The level of miR-149-5p remained indeterminate (Figure S3B). Low expression of miR-152-3p and miR-3613-3p should enhance the expression of HSP70 (HSPA1B), HSP90

FIGURE 3 Unsupervised clustering of most differentially expressed miRNAs in dendritic cells of different HBV patients groups. Unsupervised hierarchical clustering of differentially expressed miRNAs in DCs of various patients groups to show distinct patterns of up- and downregulation. The columns represent the analyzed samples, while the rows represent the miRNAs (shown on the right). The miRNA clustering tree is shown on the left and the sample groups appear at the top. The relative miRNA expression is shown on red (high) to green (low) scale. DC, dendritic cell; HBV, hepatitis B virus; miRNA, micro RNAs

(HSP90AA1), and calnexin (Figure S3B). Our RT-qPCR results confirmed elevated expression of miR-7-1-3p, miR-192-5p, and miR-195-5p and miR-32-5p and low levels of miR-3613-3p (Figure 5B). Further, all miRNA target genes except for CALR were found to be downregulated including *HLA-A*, *HLA-B*, *HLA-C*, *HSPA1B*, *HSPA8*, *HSP90AA1*, *NFYA*, *CANX*, and *PSME3* (Figure 5B).

In HBeAg negative compared to a low replicative group, two miRNAs each were either up- (miR-342-3p and miR-940) or down-regulated (miR-152-3p and miR-503-3p). The levels of miR-149-5p remained indeterminate (Figure S3C). Elevated levels of miR-342-3p and miR-940 would collectively reduce HSP70 (*HSPA1B*) expression whereas low miR-503-5p levels should enhance calnexin (*CANX*) gene expression. Further, low miR-152-3p will stimulate the expression of *HLA-A* and *HSP90* (*HSP90AA1*) genes (Figure S3C). Our RT-qPCR results largely confirmed the miRNA levels as observed in microarray and regulation of the cognate target genes (Figure 5C).

Interestingly, in AVH versus immune active group, all six miRNAs, viz., miR-34a-5p, miR-130b-3p, miR-221-3p, miR-320a,

miR-324-5p, and miR-484 were upregulated (Figure S3D). The elevated levels of these miRNAs could down-regulate the expression of multiple genes which are crucial for antigen processing and presentation pathways such as, *HLA-A*, *HLA-B*, *HLA-C*, *HSP-70*, *HSP90*, *CALX* and *CALR* and impair DC function. As expected, RT-qPCR analyses confirmed the elevated levels of all six miRNA levels and downregulation of their cognate genes (Figure 5D).

4 | DISCUSSION

The phenotypic and functional impairment of DCs in CHB patients have been reported earlier by several research groups.^{5,8,19} The impaired DC function is often correlated with ineffective antiviral response and thus, viral persistence in CHB patients. The degree of liver inflammation in CHB patients is found to be inversely proportional to DC numbers in peripheral blood owing perhaps to their migration from peripheral blood to the inflammatory site in the

FIGURE 4 Bubble plot representation of the immune signaling and biochemical pathways affected by differentially expressed miRNAs in dendritic cells of hepatitis B patients. This analysis reveals the regulation of selective signaling pathways by different clusters of miRNAs under various clinico-pathological conditions associated with HBV. The X-axis shows term enrichment of miRNAs involved in targeting various pathways (shown as colored modules) while the Y-axis shows enrichment significance (p Value) of the immune signaling and biochemical pathways. HBV, hepatitis B virus; miRNA, micro RNAs

liver.^{5,20} Further, these DCs show reduced allo-stimulatory capacity and reduced IL-6, TNF- α , and IFN- α production in response to stimulation by TLR ligands.^{5,21} Since miRNAs are important regulators of immune function,²² we investigated the differentially expressed miRNAs in DCs of HBV patients.

Analysis of miRNAs in different hepatitis B patient groups, viz., immune active, low replicative, HBeAg negative, and AVH, clearly identified 19 miRNAs that could affect MHC-I and MHC-II pathways and regulate the functioning of CD4+ and CD8+ T cells and NK cells during disease progression. We observed specific downregulation of miR-615-3p, miR-2278 and miR-3681-3p in the immune active group compared to healthy controls. downregulation of miR-615-3p and miR-2278 could be an innate mechanism allowing DC proliferation aided by elevated expression of key immune regulators such as *CANX*, *CALR*, *HSPA1B*, *HSPA8*, *HSP90AB1*, *HLA-A* and *HLA-B* genes (Figure S3A) as also corroborated by gene expression analysis (Figure 5A). In fact, a low miR-2278 level is reported to correlate with enhanced calreticulin (*CALR*) gene essential for the production of MHC class I proteins.²³ Since expression of HLA antigens are critical for the cytotoxic T cell controlled immune response against viruses and other pathogens,²⁴ its dysregulation could be associated with increased risk, rapid progression, and severity of liver diseases. Importantly, there are no reports implicating miR-615-3p, and miR-3681-3p in the regulation of HLA, calnexin, caregulin, or heat shock protein.

In low replicative compared to the immune active group, there was specific upregulation of four miRNAs, viz, miR-7-1-3p, miR-32-5p,

miR-192-5p and miR-195-5p, and downregulation of miR-152-3p and miR-3613-3p (Figure S3B). All elevated miRNAs may down-regulate the above immunoregulatory genes except for *CALR*. Further, miR-192-5p is reported to regulate liver cell death during acute liver injury²⁵ whereas miR-195-5p can regulate cell growth and inhibit cell cycle by suppressing *CDK8* or *bFGF* genes.^{26,27} However, the involvement of these miRNAs in DC function will require functional validation. downregulation of miR-152-3p and miR-3613-3p in the low replicative HBV patients may induce aberrant DNA methylation and thus, gene silencing. Importantly, the miR-152 expression is frequently downregulated in HBV-related HCC tissues and inversely correlates with DNA methyltransferase 1.²⁸ Thus, restoration of miR-152-3p could have therapeutic implications in the treatment of tumors. However, the role of miR-152-3p, miR-3613-3p, and miR-149-5p in DCs needs further investigation.

In HBeAg negative versus low replicative interpretation, two miRNAs each were upregulated (miR-342-3p and miR-940) or down-regulated (miR-152-3p and miR-503-3p) impacting the expression of their cognate genes. There are no reports on the involvement of these miRNA in DC function. Interestingly, in the AVH group compared to immune active patients, levels of all six miRNAs, viz., miR-34a-5p, miR-130b-3p, miR-221-3p, miR-320a, miR-324-5p, and miR-484 were high. Dysregulation of these miRNA may result in the overexpression of protein disulfide isomerase family A, member 3 (BRp57) or beta-2-microglobulin (B2M) in the MHC-I pathway, and cathepsin B (CTSB) in the MHC-II pathway (Figure S2D). However, there are no reports implicating these miRNAs in DC dysfunction.

FIGURE 5 Validation of expression of miRNAs and their target genes in DCs of hepatitis B patients. miRNAs and their target genes were analyzed by RT-qPCR using DC RNA samples and specific primers (Tables S3 and S4). U6 and 18S ribosomal RNAs were used as internal controls for miRNAs and mRNAs respectively. Bar charts represent results of miRNAs/mRNAs in immune active versus healthy control (A), Low replicative versus immune active (B), HBe negative compared to low replicative (C) and AVH versus immune active groups (D). All experiments were performed in triplicate. Data are shown as mean $\pm$ SD. ** and * represent significance with p values $< .01$ and $< .05$ respectively. AVH, acute viral hepatitis; DC, dendritic cell; HBV, hepatitis B virus; m RNA, messenger RNA; miRNA, micro RNAs; RT-qPCR, real time quantitative reverse transcriptase polymerase chain reaction

Thus, in this study, we found the involvement of 19 miRNAs and 12 target genes in different HBV patient groups might influence key regulatory pathways and activities of DCs during HBV pathogenesis (Table S5). Most identified miRNAs were unique and seem to differentiate acute and chronic infection with and without hepatic injury. Further, the integrome analysis confirmed the regulation of unique clusters of miRNAs and their target genes that may be responsible for viral clearance or persistence. Further, these miRNAs could be used as important therapeutic targets.

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CONFLICT OF INTERESTS

The authors of this article declare no relationships with any companies whose products or services may be related to the subject matter of the article.

AUTHOR CONTRIBUTIONS

Shiv K. Sarin and Manoj Kumar designed the study and interpreted the results. Avishek K. Singh, Sheetalnath B. Rooge, and Aditi Varshney collected clinical samples, isolated RNA from dendritic cells, and validated results. Avishek K. Singh and Robert Geffers performed the microarray analyses. Madavan Vasudevan performed the bioinformatics data analyses. Avishek K. Singh and Vijay Kumar interpreted the results and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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