

## Expression of microRNA-155 correlates positively with the expression of Toll-like receptor 7 and modulates hepatitis B virus via C/EBP- $\beta$ in hepatocytes

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**SUMMARY.** Effective recognition of viral infection and successive activation of antiviral innate immune responses are vital for host antiviral defence, which largely depends on multiple regulators, including Toll-like receptors (TLRs) and microRNAs. Several early reports suggest that specific TLR-mediated immune responses can control hepatitis B virus (HBV) replication and express differentially with disease outcome. Considering the versatile function of miR-155 in the TLR-mediated innate immune response, we aimed to study the association between miR-155 and TLRs and their subsequent impact on HBV replication using both a HBV-replicating stable cell line (HepG2.2.15) and HBV-infected liver biopsy and serum samples. Our results showed that miR-155 was suppressed during HBV infection and a

subsequent positive correlation of miR-155 with TLR7 activation was noted. Further, ectopic expression of miR-155 *in vitro* reduced HBV load as evidenced from reduced viral DNA, mRNA and subsequently reduced level of secreted viral antigens (HBsAg and HBeAg). Our results further suggested that CCAAT/enhancer-binding protein- $\beta$  (C/EBP- $\beta$ ), a positive regulator of HBV transcription, was inhibited by miR-155. Taken together, our study established a correlation between miR-155 and TLR7 during HBV infection and also demonstrated *in vitro* that increased miR-155 level could help to reduce HBV viral load by targeting C/EBP- $\beta$ .

**Keywords:** C/EBP- $\beta$ , chronic, hepatitis B virus, miRNA, Toll-like receptors.

### INTRODUCTION

Despite the presence of an effective vaccine and several agents to combat hepatitis B virus (HBV), it continues to be a serious threat to human life globally affecting more than 350 million people across the world. HBV carriers

Abbreviations: BIC, B-cell integration cluster; EBP, enhancer-binding protein; HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; miRNAs, microRNAs; PAMPs, pathogen-associated molecular patterns; PDTC, pyrrolidine dithiocarbamate; PRRs, pattern recognition receptors; TLR, Toll-like receptor; UTR, untranslated region.

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might also develop progressive liver diseases, such as liver failure, liver cirrhosis and hepatocellular carcinoma, which result in more than 500 000 deaths per year [1]. The outcome of patients with HBV infection is closely related to their innate as well as adaptive immune response [2]. While the adaptive arm of the immune system against HBV has been studied extensively [3], reports relating to the role of innate immunity in HBV infection are limited.

The innate immune response is the first line of defence activated by the host against invading pathogens. Recent data reveal that activation of the innate immune system may contribute to controlling HBV infection in hepatocytes [4]. Toll-like receptors (TLRs) play a central role in initiation of the innate immune response. They are conserved pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and mediate the innate immune responses against invading pathogens

[5]. Early reports indicate that viral infection alters the expression of TLRs as well as the downstream signalling pathways [6,7]. Reports also suggested that specific Toll-like receptor (TLR)-mediated immune response can control HBV replication and express differentially with disease outcome [8–10].

Recently, microRNAs (miRNAs) have emerged as important controllers of TLR signalling [11]. Several miRNAs are induced by TLR activation [11]. miRNAs are a class of evolutionary conserved short noncoding RNAs of 19–25 nt, synthesized from larger transcripts of the eukaryotic genome by RNA polymerase II [12]. They bind to the 3'-untranslated region (UTR) of target messenger RNA to down-regulate gene expression [13]. Multiple miRNAs are induced during the innate immune response with a consensus emerging that miR-155, miR-146 and miR-21 are predominantly ubiquitous [11]. Among them, miR-21 and miR-155 are particularly important in the context of viral infection [14–17]. However, although the contribution of miR-21 in HBV pathogenesis has been studied well [14,18], reports pertaining to the role of miR-155 in HBV infection are limited. MiR-155 was first found within the B-cell integration cluster (BIC) on chromosome 21 in the human genome [19]. It was previously reported that miR-155 plays a vital role in the regulation of host antiviral immunity [11]. It regulates the production of type I interferon and thus participates in innate antiviral immune responses [20]. An earlier report in this field reveals that miR-155 activates interferons against HBV via the JAK-STAT pathway [13]. Studies have also conveyed that miR-155 is required for effector CD8+ T-cell response to viral infection [21]. Moreover, Swaminathan *et al.* [22] have clearly established that miR-155 significantly diminishes the HIV load. Therefore, possibly miR-155 might as well be effective against HBV. Thus, considering the contribution of miR-155 in antiviral defence and its versatile function in the TLR-mediated innate immune response, we hypothesize that the expression level of TLRs might modulate the expression of miR-155 in viral pathogenesis. Therefore, we aimed to study the association between miR-155 and TLRs during HBV infection and the subsequent impact of this association on HBV replication.

## MATERIALS AND METHODS

### Study subjects

Liver biopsy specimens were procured from 12 patients with chronic HBV infection. Biopsy specimens from six individuals with steatosis but with no history of HBV, HCV or HIV infections served as disease controls. These specimens were obtained from Kalinga Gastroenterology Foundation, (Cuttack, Orissa, India). Sera specimens from 14 patients infected with HBV were also procured from Kalinga Gastroenterology Foundation, (Cuttack, Orissa, India).

Seven Control serum samples were obtained from healthy voluntary donors tested negative for HIV, HBV and HCV. Signed informed consent was obtained from the patients, and the institutional ethical committee approved the study. The diagnosis of patients with CHB was conformed according to the AASLD guidelines 2009 [23].

### Construction of plasmids

#### *pCDNA3.1.BIC*

Plasmid pCDNA3.1.BIC expressing miR-155 was constructed as described earlier [24].

#### *psiCHECK2.C/EBP $\beta$ reporter construct*

Total RNA was extracted from HepG2 cells with Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA by Revert Aid first-strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania), and a 180-bp region of the 3'UTR of *C/EBP- $\beta$* , encompassing the miR-155 seed sequence CAAGACTTA, was cloned downstream of the Renilla luciferase gene, of the dual luciferase reporter plasmid psiCHECK-2. The construct was confirmed by DNA sequencing.

### Cell culture, stimulation and transfection

The hepatoma cell lines HepG2 and HepG2.2.15, respectively, were maintained in DMEM and RPMI 1640 medium, with 10% foetal bovine serum (Sigma-Aldrich, Munich, Germany) at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

HepG2 or HepG2.2.15 cells ( $5 \times 10^5$  cells) were transfected with 1  $\mu$ g of pUC19-HBV1.3 plasmid or 1  $\mu$ g of pCDNA3.1.BIC plasmid, respectively, into a six-well plate, using Lipofectamine™ 2000 (Invitrogen). HepG2 cells transfected with 1  $\mu$ g of blank pUC19 and HepG2.2.15 cells transfected with 1  $\mu$ g of blank pCDNA3.1 served as controls, respectively. Total RNA was extracted from transfected cells after 24, 48, 72 and 96 h of transfection.

For pathway screening, HepG2 cells were stimulated with 5  $\mu$ g/mL of R837 (Invivogen, San Diego, CA, USA) alone or after pretreatment with either 10  $\mu$ M pyrrolidine dithiocarbamate (PDTC) (NF- $\kappa$ B inhibitor), 2  $\mu$ M LY294002 (PI3K inhibitor), 25  $\mu$ g/mL SP600125 (a JNK inhibitor) or 10  $\mu$ M SB203580 (p38 inhibitor) from Sigma (St. Louis, MO, USA) [20]. Relative miR-155 levels were analysed within 24 h of stimulation.

### RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) from  $5 \times 10^5$  cells or from liver biopsy specimens. Total RNA from patient sera was isolated by miR-vana™ microRNA isolation kit (Ambion, Austin, USA). RNA quality and quantity were accessed by spectrophotometer and additional visualization in agarose gels.

To analyse the mRNA expression of different TLRs and C/EBP- $\beta$ , total RNA was treated with DNase and was reverse-transcribed in a 20  $\mu$ L volume with random hexamers using the Revert Aid first-strand cDNA synthesis kit (MBI Fermentas). Real-time PCR was performed in triplicates in the ABI 7000 SDS (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green (Applied Biosystems) as described [25]. The relative quantity of the target mRNA was normalized to the level of the internal control GAPDH mRNA level.

For miRNA assay, approximately, 35 ng of total RNA was reverse-transcribed in a 10  $\mu$ L volume with the TaqMan miRNA reverse-transcriptase kit (Applied Biosystems). miR-155 expression was detected by the TaqMan human miRNA assay (Applied Biosystems) from triplicates in the ABI 7000 SDS (Applied Biosystems). miR-155 was normalized by U6 as previously described [26,27].

#### Analysis of HBV properties

Cell culture media were collected after 24, 48, 72 and 96 h of transfection of HepG2.2.15 cells with pCDNA3.1.BIC. HBV DNA from culture media was extracted using the Qiagen Blood mini-kit (Hilden, Germany). HBV DNA load in culture supernatant was quantified by absolute real-time TaqMan PCR using WHO standards as described [28]. The amount of HBV RNA in transfected cells was measured at respective time points after transfection by RT-PCR as described earlier [26]. HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) levels from culture supernatant were analysed using commercial ELISA kits (Diasorin, S.P.A., Saluggia, Italy).

#### microRNA target prediction

We have used DIANA-microT (<http://diana.cslab.ece.ntua.gr/microT/>), miRanda (<http://www.microrna.org>), PicTar (<http://pictar.mdc-berlin.de/>) and TargetScan version 5.1 ([http://www.targetscan.org/vert\\_50](http://www.targetscan.org/vert_50)) to predict the miR-155 targets.

#### Luciferase assay

HepG2 cells were plated in a 96-well, opaque, flat-bottom plate. Two sets of wells were transiently cotransfected with 50 ng of the psiCHECK2.C/EBP $\beta$  reporter construct and hsa-miR-155 mimic. One of the sets was further transfected with anti-miR-155, 24 h after the first transfection and the other with all-star negative siRNA (scrambled RNA as matched controls). HepG2 cells transfected with the psiCHECK2.C/EBP $\beta$  reporter construct and all-star negative siRNA served as control. Firefly and Renilla luciferase activities were determined 48 h after first transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The experiment was executed in triplicate, and the values were normalized to firefly luciferase.

#### Western blot analysis

HepG2.2.15 cells were harvested 24, 48, 72 and 96 h of transfection with pCDNA3.1.BIC, homogenized at 4 °C and centrifuged at 13 000 g at 4 °C for 30 min. Western blot was performed with the supernatant using antibody against C/EBP- $\beta$  from Cell Signaling Technology (Danvers, MA, USA) and antibody against  $\alpha$ -tubulin from Santa Cruz Biotechnology (Santa Cruz, CA, USA) as described earlier [13]. Densitometric measurements of bands were used for quantification by integrating each peak in Image J software (NIH, Bethesda, MD, USA).

#### Cell viability assay

To evaluate cell growth and viability, MTS assay was performed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega) [29].

#### Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software v-6.0, San Diego, CA, USA). A *t*-test (unpaired, two-tailed) was used for comparison. Non-parametric statistical analysis was performed using the Mann-Whitney *U*-test for unpaired observations. Spearman was used for correlation analysis. A *P* < 0.05 value was considered statistically significant.

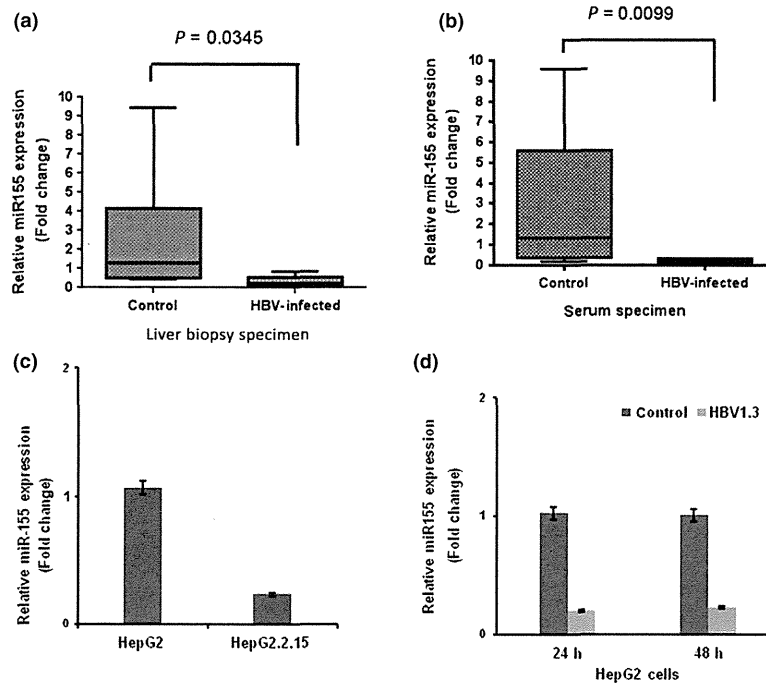
## RESULTS

### miR-155 expression is reduced during HBV infection

To acquire a preliminary idea on the expression status of miR-155 during HBV infection, we first quantified the miR-155 expression level in liver biopsy and serum samples from HBV-infected patients. We observed that miR-155 was significantly reduced in liver biopsy specimens (*P* = 0.00345) from chronic HBV patients (*n* = 12) in comparison with individuals with steatosis (*n* = 6) serving as disease controls (Fig. 1a). HBV-infected sera also exhibited a significant repression of miR-155 (*P* = 0.009) (*n* = 14) as compared to healthy controls (*n* = 6). (Fig. 1b). miR-155 level was also reduced in HepG2.2.15 cells stably replicating HBV as compared to noninfected HepG2 cells (Fig. 1c). HepG2 cells transiently transfected with HBV-expressing pUC19-HBV1.3 plasmid also displayed a repression in miR-155 expression in comparison with the control (HepG2 transfected with blank vector) (Fig. 1d).

### Expression of TLR7 correlates positively with that of miR-155 and TLR7 induces the expression of miR-155 through the NF- $\kappa$ B pathway

There are several reports suggesting that miR-155 expression can be induced by multiple TLR activation depending



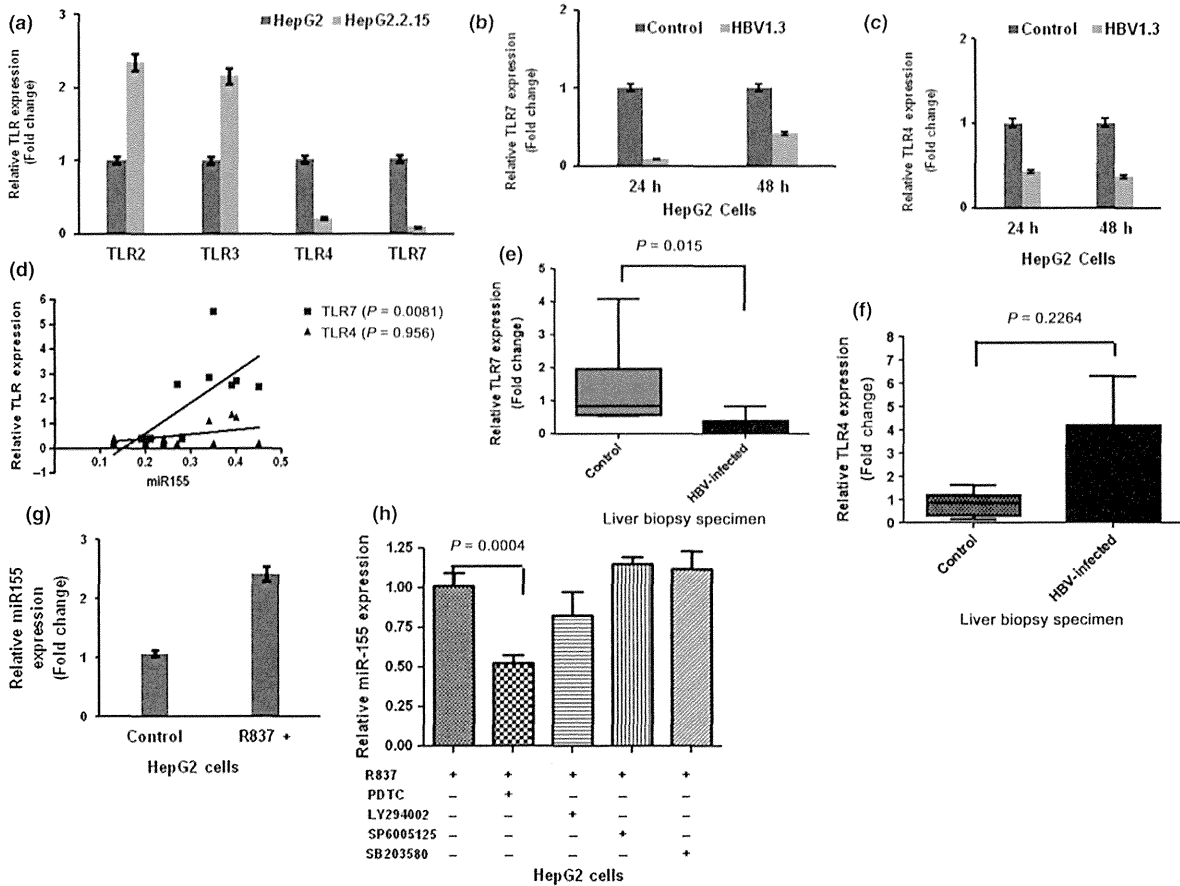
**Fig. 1** The expression of miR-155 is reduced upon HBV infection. (a) Down-regulation of miR-155 in liver biopsy specimens from patients infected with HBV ( $n = 12$ ) as compared to specimens from individuals with steatosis (non-HBV specimens with similar liver pathology) serving as disease controls ( $n = 6$ ). The data was analysed using the Mann–Whitney  $U$ -test ( $P < 0.05$ ). (b) A significant ( $P < 0.05$ ) reduction in total miR-155 expression from patient sera infected with HBV ( $n = 14$ ) compared to sera from healthy voluntary blood donors serving as controls ( $n = 6$ ). The mean miR-155 expression of non-HBV-infected (control) liver biopsy/serum specimens was arbitrarily set at 1, and the fold change in the infected liver biopsy/serum specimens was evaluated. (c) Down-regulation of miR-155 in stable HBV-replicating HepG2.2.15 hepatoblastoma cells compared to HepG2 cells serving as noninfected control. (d) A down-regulation of miR-155 in HepG2 cells transiently transfected with HBV-expressing pUC19-HBV1.3 plasmid. HepG2 cells transfected with blank vector (pUC19) served as the control. miR-155 expression in HepG2 cells/HepG2 cells transfected with blank vector was arbitrarily set at 1, and the fold change in the HepG2.2.15 cells/HepG2 cells transfected with pUC19-HBV1.3 plasmid, respectively, was evaluated. In each case, results were normalized to endogenous U6 RNA.

upon environmental stimuli [11,20]. Therefore, we tried to investigate whether the suppression in the miR-155 expression pattern was due to alteration in the expression of TLRs on account of HBV infection. We thus measured the expression of TLR2, TLR3, TLR4 and TLR7 in HepG2.2.15 cells as they were reported to be related to miR-155 (Fig 2a) [11,20]. Among them TLR4 and TLR7 were down-regulated in HepG2.2.15 cells compared to control HepG2 cells similar to miR-155, (Fig. 2a). Further, we observed the expressions of TLR4 and TLR7 in HepG2 cells transiently transfected with pUCHBV1.3. Here, also the expression of TLR4 and TLR 7 decreased with HBV infection, similar to the reduction of miR-155 (Fig. 2b,c). A statistical analysis, however, revealed a significant positive correlation in the expression pattern of miR-155 with only TLR7 in this system (Fig. 2d). We also measured the expression of TLR4 and TLR7 in liver biopsy specimens, where we found TLR7 to be significantly down-regulated

in the chronic HBV patients in comparison with steatosis patients serving as disease control, but no down-regulation could be found in the case of TLR4 (Fig. 2e,f), thus further establishing the association of TLR7 and miR-155.

To find out whether TLR7 is capable of inducing the synthesis of miR-155 in hepatocytes, we treated HepG2 cells with R837 (a TLR7 ligand) and found greater than two fold up-regulation of miR-155 after 24 h of treatment (Fig. 2g).

Stimulation by TLR ligands can lead to the activation of the MyD88/TRAF6/IRAK1/4 pathway, which in turn activates the PI3K-AKT, p38, JNK-AP1 and NF- $\kappa$ B pathways [16]. To identify the pathway that regulates miR-155 expression in the hepatocyte, we pretreated HepG2 cells with a PI3K inhibitor (LY294002), a p38 inhibitor (SB203580), a NF- $\kappa$ B inhibitor (PDTC) or a JNK inhibitor (SP600125) and then stimulated them with R837 for 24 h [20,30]. The induction of miR-155 was significantly reduced on inhibition of NF- $\kappa$ B (Fig. 2h), indicating that



**Fig. 2** Expression of TLR7 positively correlates with that of miR-155: (a) Estimation of the mRNA expression of 4 TLRs related to miR-155 synthesis (TLR2, TLR3, TLR4 and TLR7) by real-time PCR in HepG2.2.15 cells stably replicating HBV compared to control HepG2 cells shows an up-regulation of TLR2 and TLR3 expression while a down-regulation of TLR4 and TLR7 expression in HepG2.2.15 cells. Results of quantitative real-time PCR were normalized to endogenous GAPDH RNA. The expression of TLRs in HepG2 cells was arbitrarily set at 1, and the fold change in the infected HepG2.2.15 cells was evaluated. (b and c) A down-regulation of TLR4 and TLR7 in HepG2 cells transiently transfected with HBV-expressing pUC19-HBV1.3 plasmid. TLR7 expression in control (HepG2 cells transiently transfected with blank vector) at respective time points was arbitrarily set at 1, and the fold change in HepG2 cells transiently transfected with pUC19-HBV1.3 plasmid was evaluated. (d) The expression of TLR7 and TLR4 was statistically analysed with Spearman's correlation test with the expression of miR-155 in HepG2 cells transiently transfected with pUCHBV1.3 and HepG2.2.15 cells. The expression of miR-155 was significantly correlated ( $P < 0.05$ ) with that of TLR7. (e) A significant ( $P < 0.05$ ) down-regulation of TLR7 expression in liver biopsy specimens from patients infected with hepatitis B virus ( $n = 12$ ) compared to non-HBV-infected control specimens ( $n = 6$ ). (f) No significant difference in the expression pattern of TLR4 in liver biopsy specimens from patients chronically infected with HBV compared to the control. Results were normalized to endogenous GAPDH RNA and analysed using the Mann-Whitney  $U$ -test. The mean TLR7 expression in biopsy samples from control subjects was arbitrarily set at 1, and the fold change in the HBV-infected liver biopsy specimens was evaluated. TLR7 induces the expression of miR-155 through the NF- $\kappa$ B pathway. (g) Induction of miR-155 on stimulation of TLR7 by its ligand R837. HepG2 cells were treated with R837 whereby HepG2 cells treated with media served as control. (h) A significant reduction in the expression of miR-155 even after TLR7 stimulation when NF- $\kappa$ B is inhibited. HepG2 cells were treated with R837 in the presence or absence of various inhibitors. RNA was harvested before 24 h of treatment, and results were normalized to endogenous U6 RNA levels. The data obtained were from at least three independent experiments, each based on a different HepG2 preparation. The data were analysed with unpaired  $t$ -test ( $P < 0.05$ ). In both cases, the miR-155 expression in the controls was arbitrarily set at 1.

TLR7 induces miR-155 expression through the NF- $\kappa$ B pathway in hepatocytes.

#### Decrease in the hepatitis B viral load upon ectopic expression of miR-155 in HepG2.2.15 cells

Earlier *in vitro* as well as *in vivo* results have already established that stimulation of TLR7 can effectively inhibit HBV [10,31]. Interestingly, while analysing the miR-155 expression in liver biopsy specimens, we found that the HBV viral loads in the biopsies bore an inverse relationship with the expression status of miR-155 (Fig. 3). Patients numbered as 'HBV3', 'HBV4', 'HBV7', 'HBV9', 'HBV10' and 'HBV11' exhibiting high viral loads had lower miR-155 levels. Therefore, we tried to investigate whether an overexpression of miR-155 could also help in reduction of viral components. We have demonstrated that in HepG2.2.15 cells, miR-155 is significantly reduced as compared to control HepG2 cells. Transfection of HepG2.2.15 cells with miR-155-expressing pCDNA3.1.BIC (Fig. 4a) showed a steady decrease in viral DNA load from the culture supernatant from 24 h up to 96 h of transfection (Fig. 4b). The level of HBV mRNA from the transfected cells also decreased steadily as shown in Fig. 4c. HBsAg and HBeAg were detected by ELISA from culture supernatant of cells overexpressing miR-155 and respective controls. The level of both HBsAg and HBeAg showed a steady decrease from 24 h up to 96 h of transfection in comparison with their controls at respective time points (Fig. 4d,e).

#### C/EBP- $\beta$ , a miR-155 target in HBV replication

An increase of miR-155 levels in the HepG2.2.15 cells leads to a decrease in viral load and viral proteins. However, no

putative interaction site for miR-155 was found in the HBV genome by computational analysis. Therefore, it might be possible that miR-155 targets a specific host gene which participates in modulating viral replication. Using several Web-based target-predicting bioinformatics software [DIANA-microT, miRanda (microna.org), PicTar (New York, USA) and TargetScan 5.1 (Cambridge, MA, USA)], we identified C/EBP- $\beta$  (CCAAT/enhancer-binding protein- $\beta$ ), a positive regulator of HBV transcription, as a potential target of miR-155 (Fig. 5a). To confirm the prediction that miR-155 interacts with the 3'-untranslated region (UTR) of C/EBP- $\beta$ , we cloned and sequenced the 3'UTR in a dual reporter containing luciferase vector and performed the luciferase assay. The results indicated that miR-155 significantly suppressed reporter luciferase activity and subsequent anti-miR-155 treatment resulted in a 50% recovery of reporter luciferase activity (Fig. 5b).

To further verify the action of miR-155 on C/EBP- $\beta$ , we measured the expression of C/EBP- $\beta$  in HepG2.2.15 cells (exhibiting low levels of miR-155) and found that expression of C/EBP- $\beta$  was extremely high in comparison with control HepG2 cells (Fig. 5c). On the other hand, HepG2 cells transfected with puC-HBV1.3 plasmid expressed increased levels of C/EBP- $\beta$  as compared to the control (HepG2 transfected with blank vector) (Fig. 5d). Again HepG2.2.15 cells ectopically expressing miR-155 exhibited a time-dependent decrease of C/EBP- $\beta$  mRNA (Fig. 5e) and protein (Fig. 5f,g). The MTT assay confirmed that there was no significant difference in total cell viability in the control (transfected with blank vector) and miR-155-treated (transfected with pCDNA3.1.BIC) HepG2.2.15 cells (Fig. 5f). Together our results indicate that miR-155 targets C/EBP- $\beta$ , which subsequently manifest its effect on HBV replication.

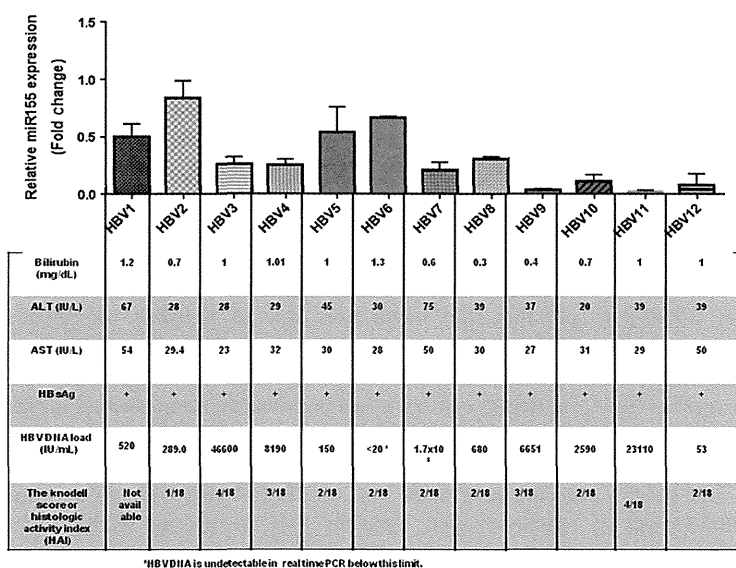
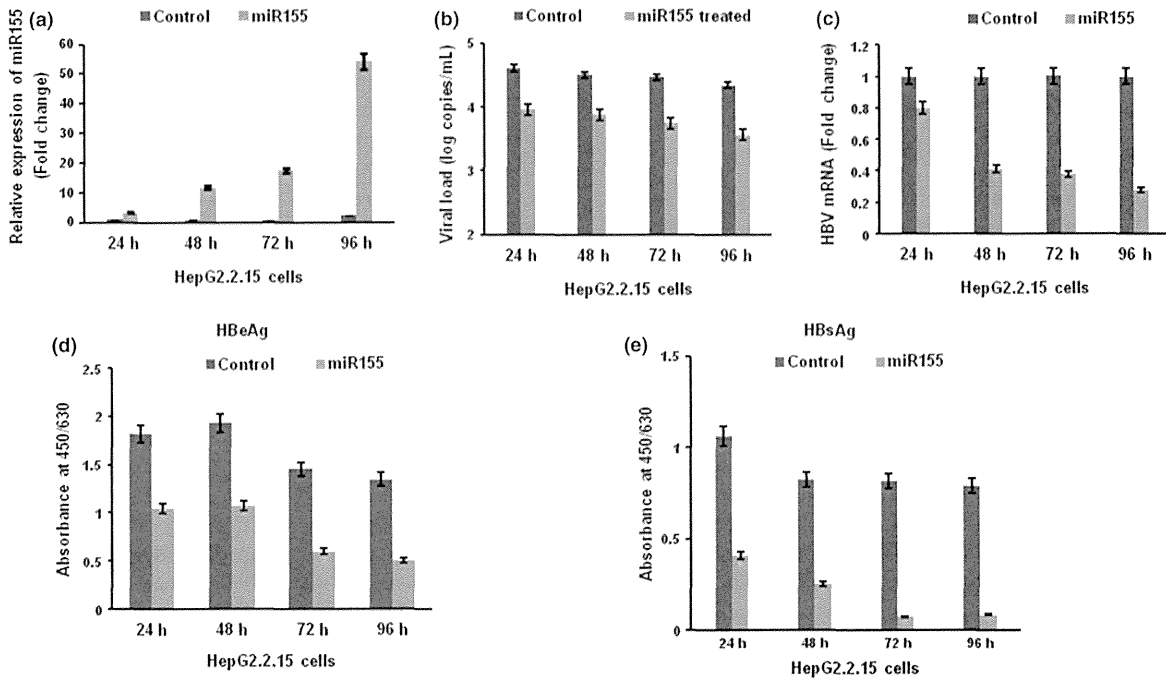


Fig. 3 miR-155 expression from liver biopsy specimens of HBV-infected patients and their corresponding clinical and virological parameters. The mean miR-155 expression of control subjects was arbitrarily set at 1, and the fold change in each of the HBV-infected patients was evaluated. U6 was used as an endogenous control. \*HBV DNA is undetectable in real time PCR below this limit.



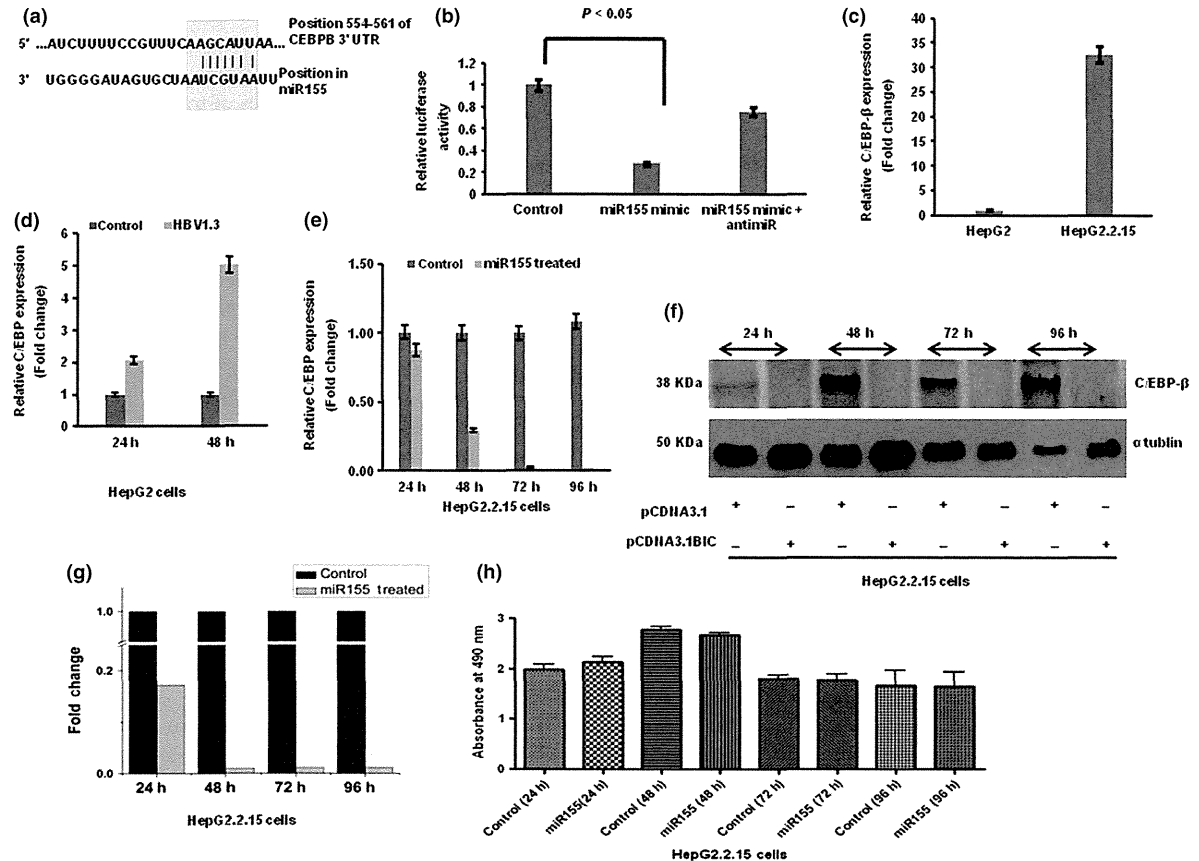
**Fig. 4** Ectopic expression of miR-155 decreases HBV DNA load, and proteins in HepG2.2.15 cells. (a) Rise in miR-155 level with time after transient transfection of HepG2.2.15 cells with pCDNA3.1.BIC and corresponding decrease in (b) HBV viral DNA load and (c) HBV mRNA level as well as (d) HBeAg and (e) HBsAg levels in the same experimental cells. HepG2.2.15 cells were transfected with pCDNA3.1.BIC plasmid, and RNA was harvested after 24, 48, 72 and 96 h of transfection. HBV DNA was isolated from the culture media of transfected cells at respective time points, and the load was assessed by an absolute real-time PCR using WHO standards. HBV RNA was also assessed by real-time PCR analysis whereby the expression of HBV mRNA levels in controls at respective time points was arbitrarily set at 1, and the fold change in the HepG2.2.15 cells transfected with pCDNA3.1.BIC plasmid was evaluated. HBsAg and HBeAg levels were determined by ELISA from culture media of transfected cells at respective time points.

## DISCUSSION

In the present study, we have found a positive correlation between TLR7 and miR-155 expression in HBV-infected liver biopsy and serum specimens as well as *in vitro* which in turn modulate HBV replication. Our principal findings are as follows: (i) HBV infection down-regulates the host miR-155 levels; (ii) TLR7 positively correlates with miR-155 during HBV infection; (iii) TLR7, on stimulation, induces the synthesis of miR-155 through the NF- $\kappa$ B pathway; (iv) ectopic expression of miR-155 in HepG2.2.15 cells reduces viral DNA as well as protein titre in a time-dependent manner by targeted suppression of C/EBP- $\beta$  which is a positive regulator of viral transcription.

In the current study, we have found that HBV down-regulates both miR-155 and TLR7 in patients as well as *in vitro*. This result has been confirmed at multiple levels which includes liver biopsy and serum specimens of HBV-infected and uninfected subjects and transient and stable HBV-replicating cell lines. The expression of miR-155 was significantly down-regulated in HBV-infected liver biopsy

specimens as compared to steatosis patients serving as disease controls. Steatosis samples have earlier been used as disease controls in HBV-related innate immune studies [32]. We have also analysed the miR-155 levels in HBV-infected serum specimens as compared to serum specimens from healthy individuals. This analysis proved that miR-155 is significantly down-regulated during HBV infection. We have also analysed this *in vitro* with HepG2 cells transiently transfected with a 1.3 $\times$  copy of HBV-containing plasmid and again found a down-regulation of miR-155 after 24 and 48 h of transfection when compared to their controls at respective time points. To overcome any complication that might arise due to transient transfection [33], we have reconfirmed our results in stable HBV-replicating HepG2.2.15 cells as compared to noninfected parental HepG2 cells as it has been widely acknowledged earlier [29]. Similarly, TLR7 was also analysed at multiple levels and has been found to be down-regulated. It has been well documented across the world that HBV induces immune tolerance upon infection [34]. Increasing studies have shown that the HBV infection impedes PRR-mediated



**Fig. 5** miR-155 targets and reduces the expression of C/EBP-β. (a) Result of TargetScan analysis showing the predicted consequential pairing of target (C/EBP-β RNA region) (top) and miRNA-155 (bottom). (b) Significant suppression of C/EBP-β promoter activity by use of miR-155 mimic in a luciferase reporter assay and recovery of 49% luciferase reporter activity after blocking miR-155 by use of anti-miR-155. The data are representative of at least 3 independent experiments, each based on a different HepG2 preparation. The data were normalized to firefly luciferase. The normalized reporter luciferase activity in the control was set at 1, and the changes in luciferase activity in miR-155 mimicked cells were significant by an unpaired *t*-test ( $P < 0.05$ ). (c) Elevated C/EBP-β mRNA expression in HepG2.2.15 cells compared to control HepG2 cells. (d) Elevated C/EBP-β mRNA expression in HepG2 cells transiently transfected with pUC-HBV1.3 plasmid as compared to control HepG2 cells (transfected with blank vector). (e) Suppression of C/EBP-β mRNA expression with time in HepG2.2.15 cells transfected with miR-155-expressing pCDNA3.1.BIC. HepG2.2.15 cells transfected with blank vector (pCDNA3.1) served as control. RNA was harvested after 24, 48, 72 and 96 h of transfection, and results were normalized to endogenous GAPDH level. In each case, (c and d) the C/EBP-β mRNA expression in controls was arbitrarily set at 1. (f and g) Down-regulation of C/EBP-β protein expression with time in HepG2.2.15 cells transfected with pCDNA3.1.BIC. (f) Result of Western blot analysis in HepG2.2.15 cells transfected with blank vector (pCDNA3.1) and HepG2.2.15 cells transfected with miR-155-expressing plasmid (pCDNA3.1BIC) at 24, 48, 72 and 96 h post-transfection using α-tubulin as the loading control and (g) normalized protein expression as quantified by Image J software from band intensities of Western blot. The band intensities of the controls (HepG2.2.15 cells transfected with blank vector) at each hour were set at 1, and the band intensities of the experimental cells (HepG2.2.15 cells transfected with pCDNA3.1.BIC) were evaluated accordingly. (h) No change in cell viability in controls and experimental HepG2.2.15 cells at each time points. The MTT assay showing that there was no significant difference in the total cell viability in the control (transfected with blank vector) and miR-155-treated (transfected with pCDNA3.1.BIC) HepG2.2.15 cells at each of the respective time points.

antiviral signalling in hepatocytes [6,7]. Pretreatment of hepatocytes with HBsAg, HBeAg or HBV virions almost completely abolishes TLR-induced capacity of antiviral

response [7]. Thus, TLR7 might be suppressed during HBV infection. Xu *et al.* [35] also reported a down-regulation of TLR7 expression in the PBMCs of CHB patients as



compared to healthy subjects [35]. Wu *et al.* [7] showed that different TLR-mediated signalling cascades were suppressed by the HBV machinery. Possibly, because of this impairment in the signalling cascade, miR-155 is suppressed along with TLR7 during HBV infection. To the best of our knowledge, this is the first study where the association between TLRs and miRNA in HBV infection has been investigated.

Previous reports indicated that TLR2, TLR3, TLR4 and TLR7 were involved in activation of miR-155 [11,20,36]. Our study clearly shows that TLR7 positively correlates with miR-155 during HBV infection. We have also shown that stimulation of TLR7 raises miR-155 through the NF- $\kappa$ B pathway in hepatocytes, which is contrary to that found in the plasmacytoid dendritic cells where the induction of miR-155 by TLR7 is via the JNK pathway [20]. However, HBV represses the expression of both TLR7 and miR-155. A recent report [37] suggested that the HBV polymerase suppressed NF- $\kappa$ B signalling and played a very important role in innate evasion which supports our results.

During HIV infection, miR-155 is raised through the TLR3 signalling pathway in macrophages [22]. However, in the current study, in HepG2.2.15 cells, we have found miR-155 to be down-regulated although the level of TLR3 is remarkably high, thus dismissing the role of TLR3 in miR-155 activation during HBV infection. Nevertheless, we have confirmed by repeated *in vitro* experimentation as well as by considering data obtained from patients' specimens, the inseparable association of TLR7 and miR-155 induction during HBV infection in hepatocytes. Thus, we have been able to establish that HBV suppresses the synthesis of miR-155 by repressing the expression of TLR7.

A major finding of the current study is that ectopic expression of miR-155 during HBV infection is capable of diminishing the viral DNA load, viral RNA titre as well as viral proteins in HepG2.2.15 cells. The HepG2.2.15 cell line is a stable HBV-producing cell line derived from the human hepatoma cell line HepG2. This cell line has been used extensively to assay the anti-HBV activities of various compounds [33]. Sells *et al.* [38] emphasize that HepG2.2.15 cells can be used in experiments designed to expand our understanding of the pathogenesis of hepatitis B and also in the design of possible antiviral drugs for treatment of chronic HBV. Therefore, we have analysed the anti-HBV effect of miR-155 in HepG2.2.15 cells.

Previously, Su *et al.* also convincingly reported that overexpression of miR-155 had an inhibitory effect on HBV transcription in HepG2 cells transiently transfected with an HBV-expressing plasmid. Based on conventional reverse-transcriptase PCR, they concluded that overexpression of miR-155 might slightly inhibit HBV X gene expression [13]. We have found that miR-155 inhibits HBV DNA, mRNA and protein and also established its molecular mechanism of action. Swaminathan *et al.* [22] reported that ectopic expression of miR-155 is capable of diminish-

ing HIV in monocyte-derived macrophages. Therefore, further *in vivo* analysis and an adequate dosage determination of miR-155 might contribute towards treatment of HIV/HBV as they share common routes of transmission.

C/EBP- $\beta$  is expressed in hepatocytes and functions in hepatocyte metabolism and proliferation [39]. Early reports have shown that it can bind and activate the HBV enhancer II [40]. It also binds to the core and surface promoter in the HBV genome to activate their transcription [41,42]. By repeated experimentation which includes bioinformatic analysis, and the luciferase assay, we have confirmed that C/EBP- $\beta$  is targeted and inhibited by miR-155. We have also found that the level of C/EBP- $\beta$  decreases with time in the same cells where we found HBV viral load to have decreased upon overexpression of miR-155 and thus establishing the role of miR-155 in inhibition of HBV via targeted repression of C/EBP- $\beta$  in hepatocytes. Conversely, the level of C/EBP- $\beta$  increases in HepG2 cells transiently transfected with pUC19-HBV1.3, where initially, we have found miR-155 expression to be suppressed and thus ascertaining the direct effect of HBV in elevating C/EBP- $\beta$  levels via miR-155 suppression. This phenomenon of elevation of C/EBP- $\beta$  might be a viral strategy to facilitate its multiplication as C/EBP- $\beta$  favours HBV [41,42].

An increasing number of studies have established that miR-155 has a pivotal role in the regulation of the host immune response. Su *et al.* [13] demonstrated that miR-155 enhances innate antiviral immunity through the JAK-STAT signalling pathway and thus inhibits HBV replication. Earlier studies have established that CD8+ T-cell-mediated apoptosis of HBV-infected hepatocytes serves as a major factor by which the human host tries to eliminate the virus during an acute response [43]. Interestingly, a study by Dudda *et al.* [21] reveals that miR-155 is essential for effector CD8+ T-cell responses to viral infection. In addition to this, our current study shows that miR-155 is capable of diminishing HBV load by targeted inhibition of C/EBP- $\beta$ . Taken together, these studies account for the multifaceted role of miR-155 against hepatitis B infection whereby on one hand, it accentuates innate and adaptive immunity against HBV and on the other hand, it suppresses HBV replication by targeted inhibition of a major transcription factor (C/EBP- $\beta$ ) which is reported to be involved in HBV replication. Recent reports show that miR-121 is already being tested in clinical trials as a treatment strategy against hepatitis C virus [44]. Thus, miR-155-based treatment might as well be developed in chronic patients whereby the idea is to generate an immune reaction as well as to trigger viral clearance in chronic patients. A major limitation of our study is the fact that it has to be tested *in vivo*. In future, *in vivo* experimental confirmation might help in the long term, to set up novel treatment strategies for effective control of chronic hepatitis B infection.

In conclusion, our study showed that miR-155 is down-regulated during HBV infection as a consequence of TLR7

suppression and if its (miR-155) level can be increased ectopically, it diminishes the total HBV load. Our findings may thus open a new avenue for the future treatment of chronically infected HBV patients.

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Article

## Reactivation of Hepatitis B Virus in Hematopoietic Stem Cell Transplant Recipients in Japan: Efficacy of Nucleos(t)ide Analogues for Prevention and Treatment

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**Abstract:** We retrospectively reviewed 413 recipients with hematologic malignancies who underwent hematopoietic stem cell transplantation (HSCT) between June 1986 and March 2013. Recipients with antibody to hepatitis B core antigen (anti-HBc) and/or to hepatitis B surface antigen (anti-HBs) were regarded as experiencing previous hepatitis B virus (HBV) infection. Clinical data of these recipients were reviewed from medical records.

We defined  $\geq 1$  log IU/mL increase in serum HBV DNA from nadir as HBV reactivation in hepatitis B surface antigen (HBsAg)-positive recipients, and also defined  $\geq 1$  log IU/mL increase or re-appearance of HBV DNA and/or HBsAg as HBV reactivation in HBsAg-negative recipients. In 5 HBsAg-positive recipients, 2 recipients initially not administered with nucleos(t)ide analogues (NUCs) experienced HBV reactivation, but finally all 5 were successfully controlled with NUCs. HBV reactivation was observed in 11 (2.7%) of 408 HBsAg-negative recipients; 8 of these were treated with NUCs, and fortunately none developed acute liver failure. In 5 (6.0%) of 83 anti-HBc and/or anti-HBs-positive recipients, HBV reactivation occurred. None of 157 (0%) recipients without HBsAg, anti-HBs or anti-HBc experienced HBV reactivation. In HSCT recipients, HBV reactivation is a common event in HBsAg-positive recipients, or in HBsAg-negative recipients with anti-HBc and/or anti-HBs. Further attention should be paid to HSCT recipients with previous exposure to HBV.

**Keywords:** HBV; hematologic malignancy; hematopoietic stem cell; reactivation

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## 1. Introduction

Hepatitis B virus (HBV) infection is one of the major health problems in the world, causing acute and chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma (HCC) [1]. The global prevalence of hepatitis B surface antigen (HBsAg) varies [2]. In Japan, the HBV carrier rate was estimated at 0.71% in 2005 [3]. It was recommended that HBV carriers requiring immunosuppressive or cytotoxic therapy should be treated with nucleos(t)ide analogues (NUCs) [2].

Occult HBV infection is characterized by the presence of HBV DNA in the liver, and in some patients also in the serum in the absence of HBsAg [4]. In general, patients with an antibody to the hepatitis B core antigen (anti-HBc) and/or to the hepatitis B surface antigen (anti-HBs) are regarded as experiencing previous HBV infection [2], which may be related to the fact that there are no universal vaccination programs against HBV in Japan. Previous studies demonstrated that transmission of HBV could occur in recipients from anti-HBc-positive donors in living related liver transplants [5,6]. Despite having anti-HBs, chemotherapy or immunosuppression, the reactivation of HBV occurs with the reappearance of HBV DNA with or without HBsAg [7]. Thus, patients with previous exposure to HBV as well as patients currently infected with HBV face the possibility of experiencing HBV reactivation and severe liver diseases. The management of these patients, and especially those with previous exposure to HBV, is still not firmly established.

HBV infection is relatively frequent in allogeneic hematopoietic stem cell transplantation (HSCT) recipients and especially in those geographical regions where HBV infection is endemic [8]. The prevalence of HBsAg-positive patients was 0.1%–1%, 10%–30% and 22.7% in the United States, Turkey and Asia–Pacific region, respectively [8,9]. It was reported that the disappearance of anti-HBs positivity was observed in recipients from serum anti-HBs-negative donors and that these recipients faced a higher risk of HBV infection was higher in these recipients [8]. Calcineurin inhibitors such as cyclosporine and tacrolimus have improved the outcomes of HSCT [10], and rituximab, a chimeric

mouse/human immunoglobulin G1 (IgG1) kappa monoclonal antibody with high affinity for CD20 antigen, which is robustly expressed by normal and malignant B cells, is also occasionally applied during the transplant treatment course [11]. However, the use of potent immunosuppressants was found to increase the probability of HBV reactivation in allogeneic HSCT recipients [12]. The balance between HBV replication and immune control after HSCT may contribute to HBV reactivation, occasionally resulting in fatal liver failure [10].

In the present study, we retrospectively examined the incidence of HBV reactivation among HSCT recipients with hematologic malignancies at our hospital in Japan, where no universal vaccination programs against HBV exist. We also evaluated the status of serum HBV markers and confirmed the effectiveness of NUCs against HBV reactivation. This study could provide new information about HBV reactivation after HSCT in HBsAg-positive and HBsAg-negative recipients.

## 2. Results

### 2.1. Hepatitis B Virus (HBV) Reactivation from Hepatitis B Surface Antigen (HBsAg)-Positive Recipients

In 120 recipients with HSCT before 2000, only 1 (1%) was HBsAg-positive and the other 119 (99%) recipients were HBsAg-negative. This single HBsAg-positive recipient experienced HBV reactivation accompanied by elevation of alanine aminotransferase (ALT) (1550 IU/L) 3 months after allogeneic HSCT for acute myeloid leukemia (AML) with cyclosporine and corticosteroid, and she was successfully treated with lamivudine and entecavir 106 months later.

In 293 recipients with HSCT in 2000 or after, 4 (1%) and 289 (99%) recipients were HBsAg-positive and -negative, respectively. Of the 4 HBsAg-positive recipients, one with autologous HSCT for multiple myeloma (MM) experienced HBV reactivation 1 month after HSCT, and he was successfully treated with lamivudine. The other 3 recipients (2 with autologous HSCT and 1 with allogeneic HSCT) were prophylactically treated with NUCs, and during the follow-up periods, up-regulation of HBV DNA levels was transiently observed, but they subsided without further treatment. In all 5 HBsAg-positive recipients, HBV DNA levels could be well controlled by the administration of NUCs.

### 2.2. HBV Reactivation from HBsAg-Negative Recipients

Of a total of 408 HBsAg-negative recipients with HSCT, 11 (2.7%) experienced HBV reactivation. In 119 HBsAg-negative recipients with HSCT before 2000 and 289 HBsAg-negative recipients with HSCT in 2000 or after, 5 (4%) and 6 (2%) recipients, respectively, experienced HBV reactivation. Among 15 (13%) HBsAg-negative recipients before 2000 and 96 (33%) HBsAg-negative recipients with autologous HSCT in 2000 or after, none had HBV reactivation. As for allogeneic HSCT recipients, 5 of 104 (5%) HBsAg-negative recipients before 2000 and 6 of 193 (3%) HBsAg-negative recipients in 2000 or after experienced HBV reactivation, respectively (no significant difference between the two periods).

### 2.3. Status of the Antibody to the Hepatitis B Surface Antigen (anti-HBs) and to the Hepatitis B Core Antigen (anti-HBc) in HBsAg-Negative Recipients with HBV Reactivation

Among 289 HBsAg-negative recipients with HSCT in 2000 or after, at least 83 (29%) had experienced previous HBV infection (Table 1). In 6 (2%) of these 289 HBsAg-negative recipients, HBV reactivation was observed: 4 (11%), 1 (2%) and 1 (2%) were observed in the groups of 35 anti-HBc-positive, 48 anti-HBc-negative/anti-HBs-positive and 49 unknown (Table 1). Cumulative HBV reactivation rates were higher in the anti-HBc-positive recipients than those in the others ( $p < 0.001$ , log-rank test; Table 1).

**Table 1.** Prevalence of antibodies to hepatitis B core antigen (anti-HBc) and to hepatitis B surface antigen (anti-HBs), and hepatitis B virus (HBV) reactivation rates in the 289 hepatitis B surface antigen (HBsAg)-negative recipients with hematopoietic stem cell transplantation (HSCT) in 2000 or after.

Status of Anti-HBc/Anti-HBs	Number of Patients	Number of HBV Reactivation	Cumulative HBV Reactivation Rates
(+)/(+)	27 (9%)	4 (11%)	9.1% at 2 years;
(+)(-)	8 (3%)	0 (0%)	14.5% at 5 years
(-)(+)	48 (17%)	1 (2%)	0.4% at 2 years;
(-)(-)	157 (54%)	0 (0%)	1.3% at 5 years
NA/NA	49 (17%)	1 (2%)	

(+), positive; (-), negative; NA, not available.

### 2.4. Characteristics of HBsAg-Negative Recipients with HBV Reactivation

Characteristics of the 11 HBsAg-negative recipients with HBV reactivation after HSCT are shown in Tables 2 and 3. Information about the donor's HBV serology, for those with HBV reactivation, was also shown in Table 3. In 10 (91%), HBV reactivation was observed within 3 years after HSCT (4–91 months, median 19 months). In 9 (82%) of the 11, HBV reactivation occurred during immunosuppression treatment. In all 11 recipients, HBsAg reappeared. Eight of 11 recipients were treated with NUCs. Among them, 4 patients received lamivudine and 4 received entecavir. Of these 8 patients, 3 achieved HBsAg to anti-HBs seroconversion 8 to 48 months after lamivudine therapy (median 11 months), and they successfully stopped the therapy without recurrence. Another recipient achieved HBsAg clearance 15 months after entecavir therapy. These 4 recipients were treated with NUCs immediately after HBV reactivation, and achieved HBV DNA  $< 2.6$  log copies/mL with a median treatment period of 2.5 months (1–7 months) (Table 3). In the remaining 4 recipients, HBV DNA was decreased in response to NUCs therapy, but it still remained positive during the observation period. There were no serious adverse events related to NUCs therapy. Fortunately, no recipients advanced to acute liver failure, and none died due to liver diseases in the present study.

**Table 2.** Characteristics of 11 HBsAg-negative recipients with HBV reactivation after hematopoietic stem cell transplantation (HSCT).

Case	Age (Years)	Gender	Type of Disease	Transplant Type	HSCT Type	Year of HSCT	Period of Immunosuppression after HSCT (Months)	Outcome	Cause of Death	Period from HSCT to Outcome (Months)
1	42	Male	AML	Allogeneic	BMT	1988	NA	Death	Unknown	18
2	44	Male	CML	Allogeneic	BMT	1994	>20	Death	Unknown	89
3	37	Male	ALL	Allogeneic	BMT	1995	8	Death	Primary disease	188
4	46	Male	AML	Allogeneic	BMT	1997	47	Death	Renal failure	47
5	40	Female	AML	Allogeneic	PBSCT	1999	48	Death	Infection	48
6	49	Female	CML	Allogeneic	PBSCT	2000	54	Alive		163
7	49	Male	MM	Autologous/ Allogeneic	PBSCT	2000	37	Death	Primary disease	105
8	22	Male	ALL	Allogeneic	PBSCT	2004	52	Alive		109
9	54	Male	MDS	Allogeneic	BMT	2008	65	Death	Esophageal cancer	66
10	53	Female	NHL	Allogeneic	BMT	2010	31	Lost to follow-up	NA	31
11	39	Male	MF	Allogeneic	PBSCT	2005	100	Alive		100

HSCT, hematopoietic stem cell transplantation; NA, not available; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; MM, multiple myeloma; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; MF, myelofibrosis; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation.



**Table 3.** HBV status in HBsAg-negative recipients who experienced HBV reactivation after hematopoietic stem cell transplantation (HSCT) and their donors.

Case	Recipients (Before HSCT)		Recipients (After HSCT)							Donor		
	anti-HBs	anti-HBc	Last Confirmed Time of HBsAg-Negativity after HSCT (Months)	Time of HBsAg-Positivity after HSCT (Months)	HBV DNA at the Time of HBsAg-Positive (log copies/mL)	Type of NUCs	Period from HBsAg Positive to NUCs Start (Months)	Outcome of HBV Status	Period from Treatment Start to Achievement of HBV Outcome (Months)	Treatment Period (Months)	anti-HBs	anti-HBc
1	NA	NA	2	8	(+)	ND	NA	HBsAg (-)	NA	NA	NA	NA
2	NA	NA	1	18	(+)	ND	NA	HBsAg (+)	19 *	NA	NA	NA
3	NA	NA	0	33	8.2	ETV	141	HBsAg (+)	12	12	(-)	(-)
4	NA	NA	0	10	(+)	ND	NA	NA	NA	NA	(-)	(-)
5	NA	NA	0	19	7.4	LAM	24	HBsAg (+)	1	1	NA	NA
6	(+)	(+)	10	28	4.5	LAM	0	anti-HBs (+)	8	>8	NA	NA
7	(+)	NA	0	31	7.3	LAM	0	anti-HBs (+)	48	9	NA	NA
8	(+)	(-)	0	4	4	LAM	0	anti-HBs (+)	11	>11	(-)	(-)
9	(+)	(+)	5	14	8.9	ETV	41	HBsAg (+)	10	>10	(-)	(-)
10	(+)	(+)	3	20	>9	ETV	4	HBsAg (+)	6	>6	(-)	(-)
11	(+)	(+)	74	91	8.6	ETV	0	HBsAg (-)	15	>15	(-)	(-)

HSCT, hematopoietic stem cell transplantation; NUCs, nucleos(t)ide analogues; anti-HBs, antibody to hepatitis B surface antigen; anti-HBc, antibody to hepatitis B core antigen; (+), positive; (-), negative; NA, not available; ND, not done; LAM, lamivudine; ETV, entecavir.; \* Period from detection of HBsAg-positive to HBV outcome.

### 3. Discussion

In the present study, the HBV reactivation rate was 100% in HBsAg-positive recipients with HSCT in the absence of HBV prophylaxis. In HBsAg-negative recipients with HSCT, 2.7% of the recipients experienced HBV reactivation. HBV reactivation was observed in 11% of HBsAg-negative and anti-HBc-positive recipients, although the number of study samples was relatively small. Notably, all recipients with HBV reactivation were successfully treated with NUCs, although some needed long-term treatment after HSCT. To add support to this conclusion, further extended follow-up periods will be needed.

In HBsAg-positive recipients, the prophylactic use of NUCs was effective for preventing HBV reactivation, supporting previous reports [13–16]. In the case of HBsAg-positive recipients, NUCs seem effective when administered with allogeneic [14] as well as with autologous HSCT [9,13]. In the present study, the 5 HBsAg-positive cases could not become NUCs-free during the follow-up periods. All patients died of primary diseases after receiving NUCs for a median period of 15 months (2–224 months). Lin *et al.* [17] reported fatal fulminant hepatitis B cases after withdrawal of prophylactic lamivudine in HSCT. Extended NUCs therapy may be safe and effective for the prevention of HBV reactivation in HBsAg-positive recipients with HSCT [18].

In HBsAg-negative recipients, we found that HBV reactivation was a rare event in both anti-HBc-negative and anti-HBs-negative recipients with HSCT. HBV reactivation occurred in almost all cases with anti-HBc and/or anti-HBs, except one case whose anti-HBc/anti-HBs status was unknown (Table 1). The status of anti-HBc and anti-HBs as well as HBsAg should be confirmed before performing HSCT, as in previous reports [19,20]. Goyama *et al.* [19] reported that the use of corticosteroids, the lack of anti-HBs in donor, and a decrease in serum anti-HBc and anti-HBs levels may predict reverse seroconversion after HSCT. Of note, HBV reactivation occurred in 82% of HBsAg-negative cases during immunosuppressive treatment in the present study. Some recent guidelines [21–23] have been recommended to start prophylactic antiviral therapy for HBsAg-negative recipients with anti-HBc and receiving HSCT. Tomblyn *et al.* [22] reported that, if the HSCT recipient is anti-HBc-positive and anti-HBs-positive, the risk of HBV reactivation is considered low during chemotherapy/conditioning, but it is thought to be higher following prolonged treatment with prednisone for graft-*versus*-host disease. They [22] also recommended that prophylactic antiviral treatment may be considered for anti-HBc-positive and anti-HBs-positive recipients before, and for 1 to 6 months after HSCT.

It is known that HBV vaccine could induce anti-HBs in a majority of vaccinees [24]. Because there are no universal vaccination programs against HBV in Japan, HBV infections are still viewed as an important issue [25]. Then, we should consider recipients with anti-HBs as having experienced HBV infection, perhaps a setting different from other countries where universal vaccination programs against HBV exist. HBV immunization of recipients of allogeneic HSCT results in a protective antibody response against HBV [26], and further studies concerning this issue are urgently required in our country.

Hui *et al.* [27] reported that it is uncertain whether late HBV-related hepatitis is due to *de novo* hepatitis B infection or transmission from donors. In our study, at least 6 of 11 donors for HBsAg-negative-recipients with HBV reactivation did not have anti-HBs and/or anti-HBc (Table 3). As

for the frequency of follow-up of these recipients, most of them did not receive HBsAg/anti-HBs checks regularly after HSCT, or regular follow-up. As recent Japanese guidelines recommended that recipients with HBsAg, anti-HBs, or anti-HBc should receive HBsAg/anti-HBs examinations regularly after HSCT, or regular follow-up [28], we plan to perform monitoring of HBV DNA monthly for 12 months after HSCT, and monthly once per 3 months after that.

In the present study, we did not observe recipients with fatal severe liver diseases. This might be because we used NUCs in the early stage of HBV reactivation. In HBsAg-negative recipients who are anti-HBc-positive and/or anti-HBs-positive, close monitoring including the measurement of HBV DNA as well as ALT levels should be mandatory. Although the intervals of this monitoring may be discussed, the present study suggested that immediate use of NUCs might be safe and effective for the prevention of HBV reactivation in HBsAg-negative recipients with HSCT.

#### 4. Patients and Methods

##### 4.1. Ethics

This work was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This retrospective study was approved by the Ethics Committee of Chiba University, Graduate School of Medicine on 31 January 2014 (No. 1754). Informed consent for participation in this study was obtained from all patients and/or their families by posting a notice in our institutes.

##### 4.2. Patients

A total of 413 recipients (mean observation period, 25 (0–309) months), treated with HSCT at Chiba University Hospital, Chiba, Japan between June 1986 and March 2013, were retrospectively reviewed for the occurrence of HBV reactivation. The patient characteristics are shown in Table 4. The recipients were divided into two groups: recipients treated with HSCT from 1986 to 1999 (before 2000), and recipients treated with HSCT from 2000 to 2013 (in 2000 or after), as nucleic acid amplification testing (NAT) of donated blood for infectious agents was introduced in Japan in October 1999, and Japanese health insurance approved the first NUC, lamivudine, for the treatment of hepatitis B recipients in September 2000.

**Table 4.** Patient characteristics in the present study.

Characteristics	Total ( <i>n</i> = 413)	1986–1999 ( <i>n</i> = 120)	2000–2013 ( <i>n</i> = 293)
Gender			
Male, <i>n</i> (%)	250 (60.5)	70 (58.3)	180 (61.4)
Female, <i>n</i> (%)	163 (39.5)	50 (41.7)	113 (38.6)
Median age, years (range)	42 (15–69)	32 (15–57)	47 (16–69)
Type of disease, <i>n</i> (%)			
AML	115 (27.8)	36 (30.0)	79 (26.7)
ALL	61 (14.8)	28 (23.3)	31 (10.6)
CML	38 (9.2)	20 (16.7)	18 (6.1)
MDS	31 (7.5)	9 (7.5)	22 (7.5)

Table 4. Cont.

Characteristics	Total (n = 413)	1986–1999 (n = 120)	2000–2013 (n = 293)
Lymphoma	84 (20.3)	18 (15.0)	68 (23.2)
Plasma cell dyscrasia * <sup>1</sup>	61 (14.8)	0 (0)	61 (20.8)
Aplastic anemia	16 (3.9)	9 (7.5)	7 (2.4)
Others * <sup>2</sup>	7 (1.7)	0 (0)	7 (2.4)
Transplant type, n (%)			
Autologous	114 (27.6)	15 (12.5)	99 (33.8)
Allogeneic	299 (72.4)	105 (87.5)	194 (66.2)
For Allogeneic-SCT			
Donor source, n (%)			
Related	126 (42.1%)	59 (56.2%)	67 (34.5%)
Unrelated	135 (45.2%)	46 (43.8%)	89 (45.9%)
Unrelated cord blood	38 (12.7%)	0 (0%)	38 (19.6%)

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; SCT, stem cell transplantation. \*<sup>1</sup> Plasma cell dyscrasia included multiple myeloma, AL amyloidosis and POEMS syndrome; \*<sup>2</sup> Others included primary myelofibrosis, chronic active EB virus infection and chronic eosinophilic leukemia.

#### 4.3. Serological Examination

HBsAg, anti-HBs and anti-HBc were determined by ELISA or chemiluminescent enzyme immunoassay (CLEIA) [29]. Depending on the time-point during this retrospective study, HBV DNA was measured by Roche Amplicore PCR assay (detection limit: 2.6 log IU/mL), COBAS TaqMan HBV test v2.0 (detection limit: 2.0 log IU/mL) (Roche Diagnostics, Basel, Switzerland), or PCR methods. If needed, we performed in-house nested PCR [29]. All serological tests were performed at the Central Laboratory of Chiba University Hospital.

HBsAg was measured in all recipients before and after HSCT. Among 120 recipients with HSCT before 2000, anti-HBs and anti-HBc were measured in only 10 (8%) recipients. Among the patients with HSCT performed in 2000 or after, the recipients were further divided into two groups: 2000–2005 and 2006–2013. Of 121 recipients with HSCT in 2000–2005, anti-HBs and anti-HBc were measured in 76 (63%) recipients. Finally, among 172 recipients with HSCT in 2006–2013, anti-HBs and anti-HBc were measured in 168 (98%) recipients (Figure 1). HBsAg, anti-HBs and anti-HBc were measured for screening in almost all recipients after the Japanese Health and Labor Sciences Research Group for “Clarification of current status for reactivation of hepatitis B virus associated with immunosuppressants and antineoplastics and establishment of preventive measures” started a registry in 2009 for HBV-infected patients with hematopoietic malignancies [28].