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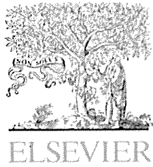
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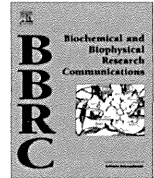

A novel TK-NOG based humanized mouse model for the study of HBV and HCV infections

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ABSTRACT

The immunodeficient mice transplanted with human hepatocytes are available for the study of the human hepatitis viruses. Recently, human hepatocytes were also successfully transplanted in herpes simplex virus type-1 thymidine kinase (TK)-NOG mice. In this study, we attempted to infect hepatitis virus in humanized TK-NOG mice and urokinase-type plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice. TK-NOG mice were injected intraperitoneally with 6 mg/kg of ganciclovir (GCV), and transplanted with human hepatocytes. Humanized TK-NOG mice and uPA/SCID mice were injected with hepatitis B virus (HBV)- or hepatitis C virus (HCV)-positive human serum samples. Human hepatocyte repopulation index (RI) estimated from human serum albumin levels in TK-NOG mice correlated well with pre-transplantation serum ALT levels induced by ganciclovir treatment. All humanized TK-NOG and uPA-SCID mice injected with HBV infected serum developed viremia irrespective of lower replacement index. In contrast, establishment of HCV viremia was significantly more frequent in TK-NOG mice with low human hepatocyte RI (<70%) than uPA-SCID mice with similar RI. Frequency of mice spontaneously in early stage of viral infection experiment (8 weeks after injection) was similar in both TK-NOG mice and uPA-SCID mice. Effects of drug treatment with entecavir or interferon were similar in both mouse models. TK-NOG mice thus useful for study of hepatitis virus virology and evaluation of anti-viral drugs.

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1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 and 170 million people are infected with HBV and HCV, respectively [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and potentially death due to liver failure and hepatocellular carcinoma [3]. Although the chimpanzee is a useful animal model for the study of HBV and HCV infection, there are ethical restrictions and hampered by the high financial cost on the use of this animal. The immunodeficient mice with a urokinase-type plasminogen activator (uPA) transgene [4,5] or a targeted disruption of the murine fumaryl acetoacetate hydrolase (FAH) [6–10] were shown to be excellent recipients for human hepatocyte. These small animal models are available for hepatitis viruses infection [4,11], and are useful for the study of HBV and HCV biology [12–14]. However, there are disadvantages that limit the utility of this model for many applications, including excessive mortality [9].

Recently, human hepatocytes were successfully transplanted into severely immunodeficient NOG mice with the herpes simplex virus type-1 thymidine kinase (HSVtk) expressing in mouse hepatocytes (TK-NOG) [15]. Mouse liver cells expressing HSVtk

Abbreviations: ALT, alanine aminotransferase; GCV, ganciclovir; HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; HSVtk, herpes simplex virus type-1 thymidine kinase; IFN, interferon; PegIFN-alpha, pegylated interferon-alpha; RI, repopulation index; RT-PCR, reverse transcript-polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator.

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were ablated after a brief exposure to ganciclovir (GCV), and transplanted human hepatocytes were stably maintained within the mouse liver without exogenous drug administration [15]. The analyses of drug interactions and pharmacokinetics have previously been reported using TK-NOG mice transplanted with human hepatocytes [15–18]. In the present study, we succeeded in infecting human hepatocyte-transplanted TK-NOG mice with HBV and HCV and showed that this mouse model is as useful as the uPA/SCID model for the study of hepatitis viruses.

2. Materials and methods

2.1. Animal treatment

TK-NOG mice were purchased from Central Institute for Experimental Animals (CIEA, Kawasaki, Japan). Eight-weeks-old mice were injected intraperitoneally with 6 mg/kg of GCV twice a day. After two days, mice were re-injected with the same amount of GCV. Seven days after 1st GCV injection, mice were transplanted with 1 or 2 $\times$ 10^6 of human hepatocytes obtained from human hepatocyte transplanted uPA–SCID chimeric mice by collagenase perfusion method by intra-splenic injection. Transplanted human hepatocytes used in this study were obtained from a same donor. One week after the first GCV treatment, serum alanine aminotransferase (ALT) levels were measured (Fuji DRI-CHEM, Fuji Film, Tokyo, Japan). Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentration of human serum albumin (HSA), which correlated with the human hepatocyte repopulation index (RI) [15], was measured as previously described [5]. Generation of the uPA/SCID mice and transplantation of human hepatocytes were performed as described previously [5,12,19]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

2.2. Human serum samples

Human serum samples containing high titers of either genotype C HBV (5.3 $\times$ 10^6 copies/mL) or genotype 1b HCV (2.2 $\times$ 10^6 copies/mL) were obtained from patients with chronic hepatitis who provided written informed consent. The individual serum samples were divided into small aliquots and stored separately in liquid nitrogen until use. Mice were injected intravenously with 50 μL of either HBV- or HCV-positive human serum. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institutional review committee.

2.3. Quantitation of HBV and HCV

DNA and RNA extraction and quantitation of HBV and HCV by real-time polymerase chain reaction (RT-PCR) were performed as described previously [12,13,19]. Briefly, DNA was extracted using SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL H_2O, and RNA was extracted from serum samples using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HBV DNA and HCV RNA was performed using Light Cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limits of real-time PCR for HBV DNA and HCV RNA are 4.4 and 3.5 log copies/mL, respectively.

2.4. Histochemical analysis of mouse liver

Liver specimens of HBV-infected TK-NOG mice were fixed with 10% buffered-paraformaldehyde and embedded in paraffin blocks for histological examination. Hematoxylin-eosin and immunohistochemical staining using antibodies against HSA (Bethyl Laboratories Inc., Montgomery, TX) and hepatitis B core antigen (HBc-Ag) (DAKO Diagnostika, Hamburg, Germany) were performed as described previously [12].

2.5. Treatment with antiviral agents

Mice were treated with antiviral agents eight weeks after HBV or HCV infection, by which time stable viremia had developed. HBV-infected mice were administered either food containing 0.3 mg of entecavir/kg of body weight/day or daily intramuscular injections with 7000 IU/kg of IFN-alpha (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). HCV-infected mice were administered intramuscular injection with either 1000 IU/kg of IFN-alpha daily or 10 μg/kg of PegIFN-alpha-2a (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) twice a week for three weeks.

2.6. Statistical analysis

Differences in HSA levels between TK-NOG mice and uPA–SCID mice, and incidence of infection between highly and poorly repopulated mice were examined for statistical significance using the Mann–Whitney *U*-test.

3. Results

3.1. Correlation between serum ALT level after GCV administration and the human hepatocyte index in TK-NOG mice

We analyzed the correlation between serum ALT levels after GCV injection and the human hepatocyte RI using 194 TK-NOG mice. Seven days after GCV injection when serum ALT levels had reached maximum levels [15], mice were transplanted with human hepatocytes. After transplantation of human hepatocytes, serum concentrations of HSA increased and reached plateau at 6–8 weeks. Serum ALT levels one week after GCV administration and HSA levels 8 weeks after hepatocyte transplantation showed a positive correlation, indicating that the higher serum ALT level, the higher the RI (Fig. 1A). HSA levels 8 weeks after human hepatocyte transplantation in TK-NOG mice were lower than in uPA–SCID mice (Fig 1B), which indicates that mice livers were more efficiently replaced with human hepatocytes in uPA–SCID mice than in TK-NOG mice.

3.2. Infection with hepatitis viruses in humanized TK-NOG mice and uPA–SCID mice

Eight weeks after human hepatocyte transplantation, TK-NOG mice and uPA–SCID mice with HSA levels over 1.0 mg/mL were inoculated with either HBV- or HCV-positive human serum samples. Eight weeks after injection, the frequency of the development of viremia was compared between the mice with lower (<70%) and higher ($\geqslant$70%) human hepatocyte RI. 70% of RI corresponds to 5.4 and 6.3 mg/dl of serum HAS in TK-NOG mice and uPA–SCID mice, respectively [5,15]. All humanized TK-NOG and uPA–SCID mice inoculated with HBV developed viremia 8 weeks after injection, irrespective of the RI (Fig. 2A). Incidence of HCV viremia was also high in TK-NOG mice regardless of the RI. In contrast, the frequency of HCV viremia was much lower in uPA–SCID mice with the RI. Only 20% (1 of 5) of uPA–SCID mice with low RI became

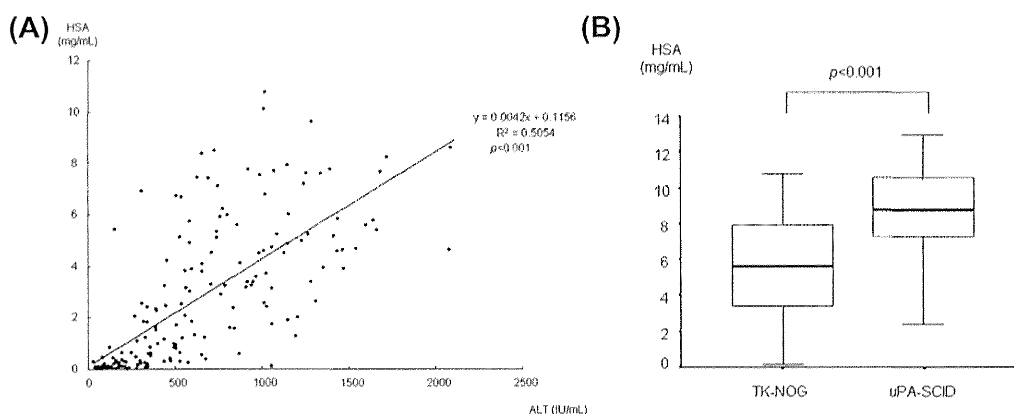

Fig. 1. Human hepatocyte repopulation index in humanized mice. Serum alaninaminotransferase (ALT) levels in TK-NOG mice were measured one week after ganciclovir treatment. Human serum albumin (HSA) levels were measured eight weeks after transplantation of human hepatocytes. (A) Correlation between serum ALT level after ganciclovir administration and human hepatocyte repopulation index in TK-NOG mice. Points represent single mouse measurements. r (Spearman rank) and P value are shown. (B) HSA levels in TK-NOG mice and uPA–SCID mice. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively.

positive for HCV, whereas 94.3% (50 of 53) of mice with high RI became positive ($p = 1.07 \times 10^{-6}$). Serum viral titers gradually increased in mice that developed viremia. Eight weeks after infection, HBV DNA and HCV RNA titers increased to approximately 8 and 6 log copies/mL, respectively in both TK-NOG and uPA–SCID mice (Fig. 2B). Viremia levels were slightly higher in uPA–SCID mice than TK-NOG mice, probably due to higher human hepatocyte RI (HSA levels) in uPA–SCID mice. In HBV-infected TK-NOG mice, histological analysis showed that hepatocytes positive for HSA were also positive for HB core antigen (Fig. 2C), which is in line with our previous findings using uPA–SCID mice [12].

3.3. The effect of antiviral agents on hepatitis virus-infected humanized mice

We analyzed the effect of antiviral agents on HBV- and HCV-infected humanized mice. Eight weeks after HBV-infection, 2 humanized TK-NOG mice were orally administrated 0.3 mg/kg day of entecavir, and 2 other mice received intramuscular injections with 7000 IU/g of IFN-alpha daily for 3 weeks. Both treatments resulted in a rapid reduction of mouse serum HBV DNA titers (Fig. 3A). Two HCV-infected humanized TK-NOG mice were administrated IFN-alpha daily, and 2 other mice received PegIFN-alpha-2a injections twice a week for 3 weeks. Both treatments resulted in a reduction of HCV RNA titers in mouse serum. The effects of these antiviral agents on HBV and HCV in TK-NOG mice were similar to those in uPA–SCID mice (Fig. 3B).

3.4. Incidence of unexpected death

The incidence of unexpected death is high in human hepatocyte chimeric uPA–SCID mice [20]. Incidence of unexpected death in the early stages of viral infection (within 8 weeks of viral infection) was similar between TK-NOG mice and uPA–SCID mice (6.3% vs 10.6%, $p = 0.465$) (Fig. 4).

4. Discussion

Human hepatocyte chimeric mice are valuable tool for hepatitis virology and drug assessment [12–14]. To establish human hepatocyte chimerism, two conditions are necessary: immunodeficiency and mouse-specific liver cell damage. For immune deficiency, SCID mice [4,5,12–14,20], NOG mice [8,21] and RAG-2 deficient mice [6,9,10] have been reported. We previously reported that the level of immunodeficiency in SCID mice, which are the most weakly immunodeficient of the three types, is sufficient to prevent rejection of transplanted human hepatocytes [5]. However, preventive treatments for human liver cell rejection via mice NK cells, such as an anti-asialo GM1 antibody, are necessary in SCID mice [5].

To evoke mouse liver cell injury, uPA and FAH transgene techniques were used [4–10]. Recently, successful human liver cell transplantation to TK-NOG mice in the absence of ongoing drug treatment after a brief exposure to a non-toxic dose of GCV has been reported [15]. We thus attempted to use TK-NOG mice to establish high levels of replacement with human hepatocytes and tried to infect hepatitis viruses.

In this study, we transplanted human hepatocytes to 194 TK-NOG mice and analyzed whether elevated serum ALT levels, which results from liver damage caused by GCV exposure, reflects HSA levels, as it is known that HSA levels are correlated with the human hepatocyte RI and can serve as a surrogate measure [15]. We found a positive correlation between ALT and HSA levels (Fig. 1A), indicating that higher levels of liver damage are associated with establishment of higher levels of repopulation of the liver with human hepatocytes. As the human hepatocyte RI obtained in this study using TK-NOG mice is lower than in uPA–SCID mice (Fig 1B), dose escalation of GCV or alternative treatment timing might result in more highly repopulated mice.

We infected humanized TK-NOG mice with hepatitis viruses and compared infection rates and serum viral titers with humanized uPA–SCID mice. HBV inoculation resulted in development of viremia without regard for the human hepatocyte replacement index in both TK-NOG mice and uPA–SCID mice (Fig. 2A). Incidence of HCV viremia was also high in TK-NOG mice regardless of HSA levels, whereas HCV viremia was infrequent in uPA–SCID mice with low HSA levels. These results are consistent with those of Vanwolleghem et al. [20] who showed, using a large number of human hepatocyte chimeric uPA–SCID mice, that an HSA level well above 1 mg/mL is important for successful HCV infection. The reason for the higher infection rate in TK-NOG mice with low human hepatocyte RI in this study is unknown. Although the level of immunodeficiency is higher in TK-NOG mice, it is difficult to conclude that this difference in immunodeficiency alone is responsible for the enhanced HCV infection rate. Although some studies have

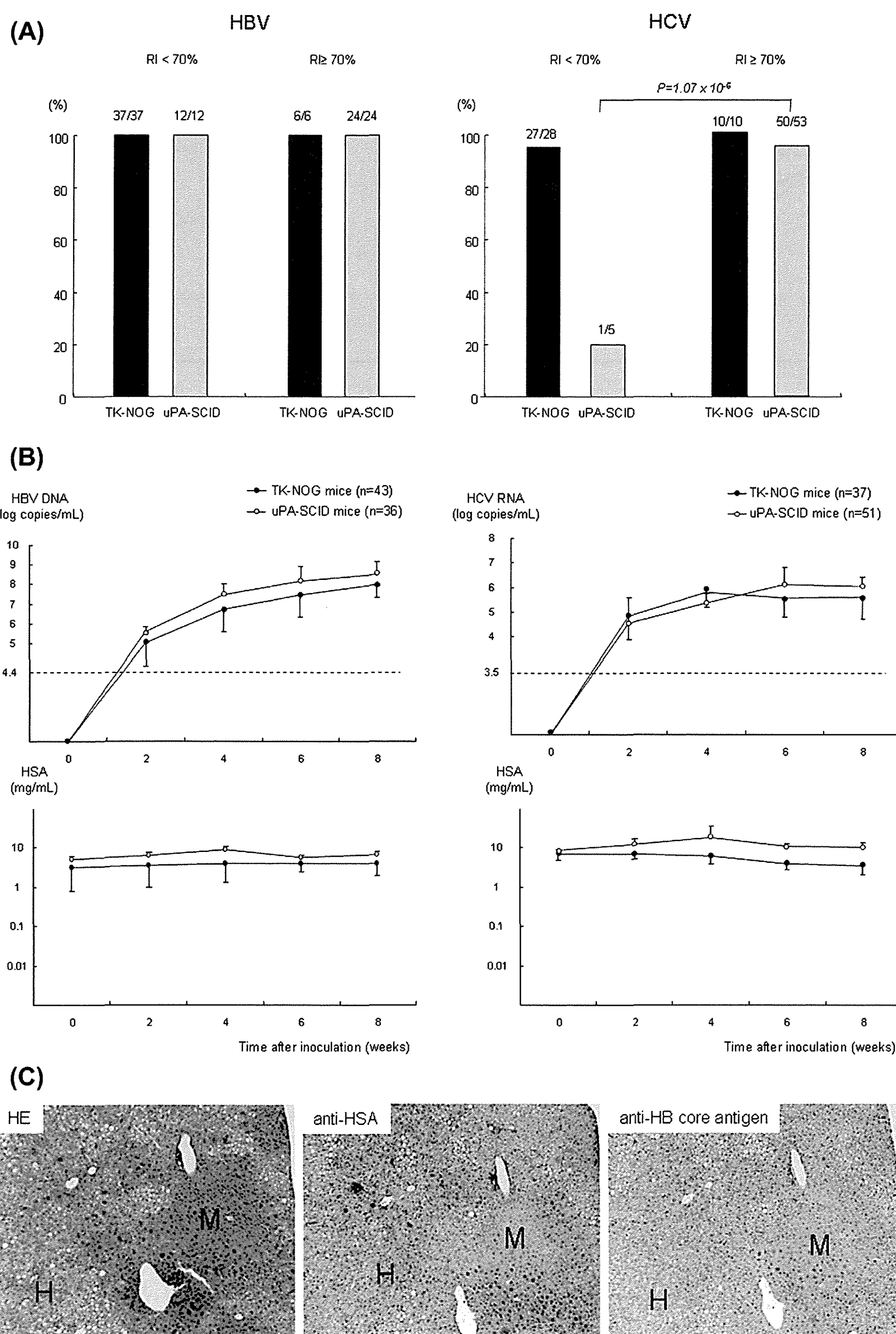

Fig. 2. Hepatitis viruses infection in chimeric mice. (A) Eight weeks after human hepatocyte transplantation, mice with serum HSA level over 1 mg/mL were inoculated with HBV- or HCV-positive human serum samples. Percentages of mice that became positive for HBV DNA (left panel) or HCV RNA (right panel) 8 weeks after inoculation according to human hepatocyte repopulation index (RI) in TK-NOG mice and uPA–SCID mice are shown. 70% of RI corresponds to 5.4 and 6.3 mg/dl of serum HAS in TK-NOG mice and uPA–SCID mice, respectively. (B) Changes in serum titers of HBV DNA (left panel) and HCV RNA (right panel) (upper panels) and HSA levels (lower panels) of TK-NOG mice and uPA–SCID mice. The horizontal dashed lines represent the lower detection limit of HBV DNA and HCV RNA (4.4 and 3.5 log copies/mL, respectively). (C) Histochemical analysis of liver samples obtained from HBV-infected TK-NOG mice. Hematoxylin-eosin staining (HE) and immunohistochemical staining using monoclonal antibodies against HSA and HB core antigen are shown. Regions are shown as human (H) and mouse (M) hepatocytes, respectively (Original magnification 100×).

reported structural differences between wild type and chimeric mice [22,23], the influence of such structural differences on HCV infectivity remains to be determined.

Human hepatocyte transplanted uPA–SCID mice are useful for evaluating antiviral agents [12–14]. In this study, we analyzed the efficacy of antiviral agents such as entecavir, IFN-alpha and

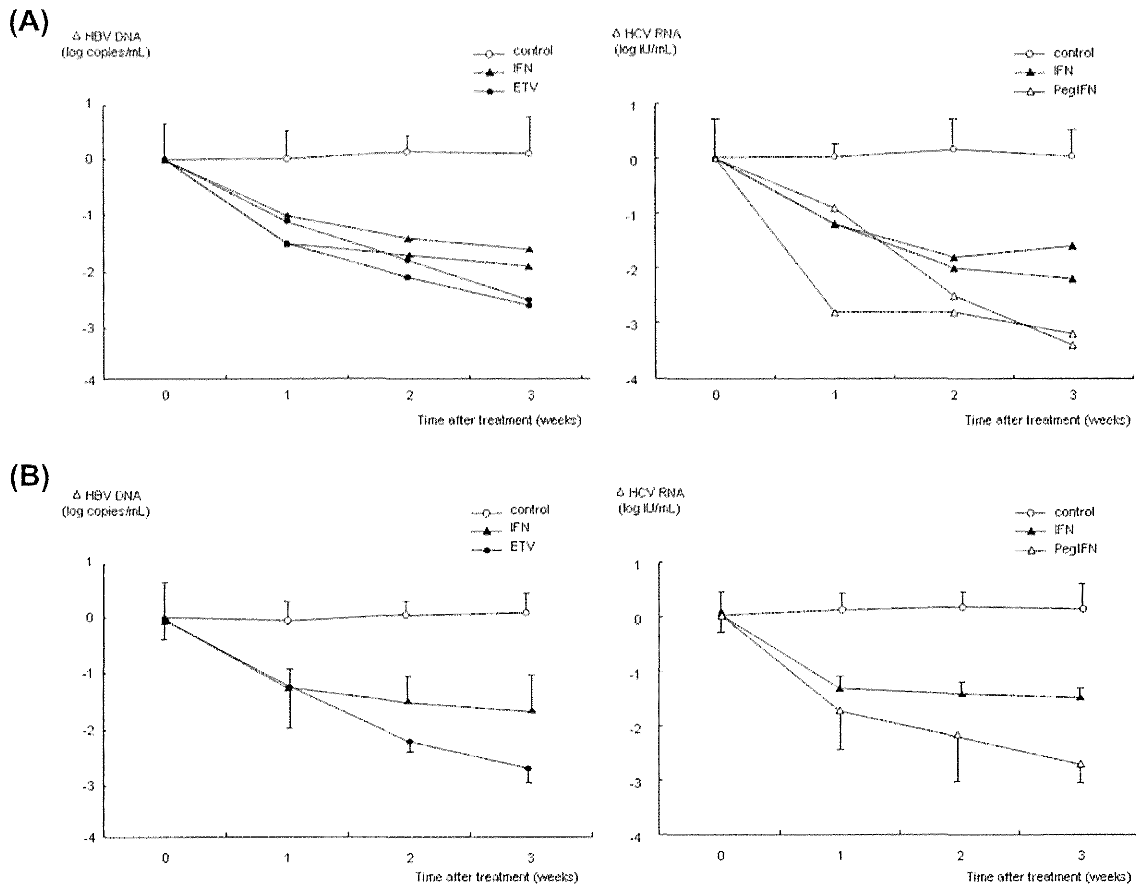

Fig. 3. Reduction of serum viral titers in mice treated with anti-viral agents. (A) HBV- (left panel) or HCV-infected (right panel) TK-NOG mice were treated with entecavir, interferon (IFN)-alpha or PegIFN-alpha-2a. Control: HBV- and HCV-infected mice without antiviral treatment. (B) HBV- (left panel) or HCV-infected (right panel) uPA–SCID mice were treated with entecavir, IFN-alpha or PegIFN-alpha-2a. Data are shown using the mean ± SD ($n = 4$).

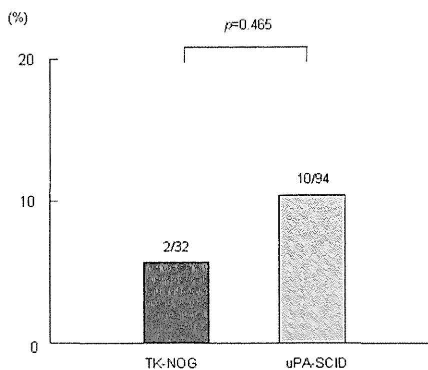

Fig. 4. Frequency of unexpected death within 8 weeks in mice. The numbers of sudden deaths occurring within 8 weeks of viral infection in TK-NOG mice and uPA–SCID mice are shown as bars.

PegIFN-alpha using HBV- and HCV-infected TK-NOG mice and compared them with uPA–SCID mice (Fig. 3). The results showed that both mouse models are equally useful for evaluation of anti-viral drugs.

Human hepatocyte chimeric uPA–SCID mice are weak and prone to unexpected death [20], and this limitation appears to apply to TK-NOG mice as well. Incidence of unexpected death in the early stages of viral infection was not significantly different between TK-NOG mice and uPA–SCID mice (Fig. 4). The cause of these unexpected deaths is unknown. Further study is necessary to develop a more robust and easy to manipulate animal model.

In summary, we established a hepatitis virus infection mouse model using the human hepatocyte transplanted TK-NOG mouse. This model is useful for the study of hepatitis virology and evaluation of antiviral agents.

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Circulating MicroRNA-22 Correlates with MicroRNA-122 and Represents Viral Replication and Liver Injury in Patients with Chronic Hepatitis B

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Hepatitis B virus (HBV) infection is associated with increased expression of microRNA-122. Serum microRNA-122 and microRNA-22 levels were analyzed in 198 patients with chronic HBV who underwent liver biopsy and were compared with quantitative measurements of HBsAg, HBeAg, HBV DNA, and other clinical and histological findings. Levels of serum microRNA-122 and microRNA-22 were determined by reverse transcription-TaqMan PCR. Serum levels of microRNA-122 and microRNA-22 were correlated ($R^2 = 0.576$; $P < 0.001$), and both were elevated in chronic HBV patients. Significant linear correlations were found between microRNA-122 or microRNA-22 and HBsAg levels ($R^2 = 0.824$, $P < 0.001$ and $R^2 = 0.394$, $P < 0.001$, respectively) and ALT levels ($R^2 = 0.498$, $P < 0.001$ and $R^2 = 0.528$, $P < 0.001$, respectively). MicroRNA-122 levels were also correlated with HBV DNA titers ($R^2 = 0.694$, $P < 0.001$ and $R^2 = 0.421$, $P < 0.001$). Levels of these microRNAs were significantly higher in HBeAg-positive patients compared to HBeAg-negative patients ($P < 0.001$ and $P < 0.001$). MicroRNA-122 levels were also lower in patients with advanced liver fibrosis ($P < 0.001$) and lower inflammatory activity ($P < 0.025$). These results suggest that serum micro-RNA levels are significantly associated with multiple aspects of HBV infection. The biological meaning of the correlation between microRNA-122 and HBsAg and should be investigated further. ***J. Med. Virol. 85:789–798, 2013.***
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KEY WORDS: HBsAg; histological activity; inflammation; microRNA

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Abbreviations: ALT, alanine transaminase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR-122, microRNA-122; miR-22, microRNA-22; PCR, polymerase chain reaction.

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INTRODUCTION

Hepatitis B virus (HBV) is a small enveloped virus with a partially double-stranded 3.2 kb DNA genome belonging to the Hepadnaviridae family [Fields et al., 2007]. Chronic HBV infection is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [Beasley et al., 1981]. More than 350 million people are persistent carriers of HBV and many may progress to chronic liver disease [Lavanchy, 2004; McMahon, 2009].

MicroRNAs are a class of naturally occurring short non-coding RNAs that regulate the expression of a wide range of genes and play an important role in various biological functions including cell differentiation, development, immune responses, metabolism, and carcinogenesis. Circulating microRNAs are bound to Ago2 and remain in the serum for an extended period of time [Blumberg et al., 1965; Bala et al., 2009]. Liver damage ultimately results in alteration of hepatic and serum microRNA expression profiles [Bala et al., 2009]. Hepatocellular carcinoma-associated expression profiles have been reported by a number of laboratories [Murakami et al., 2006; Ji et al., 2009; Ura et al., 2009; Gao et al., 2011; Hou et al., 2011; Mizuguchi et al., 2011], but microRNA expression profiles may differ based on etiology, including differences among patients infected with HBV compared with patients infected with hepatitis C virus (HCV). HBV infection disrupts pathways involved in signal transduction, DNA damage, and cell death, whereas HCV infection tends to disrupt pathways involved in lipid metabolism, cell cycle regulation, and immune response [Ura et al., 2009].

Many of these cellular changes are mediated by changes in microRNA expression, suggesting that analysis of microRNA expression may improve understanding of HBV pathogenesis and uncover new avenues for risk assessment and therapy. A number of microRNAs associated with HBV infection have been reported [Bala et al., 2009], but in most cases little is known about the biological roles of the identified microRNAs. In this study, two microRNAs, microRNA-122 (miR-122) and microRNA-22 (miR-22), were examined as possible biomarkers for association with chronic HBV infection. miR-122 was selected due to its strong expression in the liver and central role in liver function, and because it directly suppresses HBV replication by binding to viral RNA [Qiu et al., 2010; Chen et al., 2011]. Serum miR-122 has been reported as a biomarker for various liver injuries and is correlated with levels of ALT, HBV DNA, and HBsAg [Zhang et al., 2010; Waidmann et al., 2012]. Circulating miR-122 is elevated in patients with chronic hepatitis B, especially in patients positive for HBeAg [Xu et al., 2010; Ji et al., 2011; Qi et al., 2011; Zhou et al., 2011; Waidmann et al., 2012]. miR-22 was selected for this study because it is also highly expressed in the liver and has been implicated in HCC and liver failure in patients infected with HBV [Ji et al., 2011; Jiang et al., 2011; Xu et al., 2011]. miR-22 is described in the literature both as a tumor-suppressor [Xu et al., 2011] and as a micro-oncogene [Liu et al., 2010] due to its central role in targeting multiple genes involved in determining cell fate, including PTEN [Liu et al., 2010], p21 [Tsuchiya et al., 2011], Mat1a and Mthfr [Koturbash et al., 2011], and senescence-associated transcripts CDK6, SIRT1, and Sp1 [Xu et al., 2011]. miR-22 also targets estrogen receptor alpha [Pandey and Picard, 2009], which compromises the protective effects of estrogen and leads to up-regulation of IL-1α in hepatocytes under conditions of oxidative stress, such as that caused resulting from activity of the HBx protein [Jiang et al., 2011]. HBV also evades senescence through hypermethylation of p16 and transcriptional interference in components of the stress-induced senescence pathway [Kim et al., 2010]. Changes in miR-22 expression may, therefore, reflect cellular changes leading to suppression of senescence and indicate an increased risk of dysplasia.

Because of their prominent roles in the liver and association with HBV infection, serum microRNA levels of miR-122 and miR-22 were compared between healthy individuals and patients with chronic HBV infection, and correlation with clinical and histological parameters were examined.

MATERIALS AND METHODS

Study Patients

One hundred and ninety-eight patients with chronic hepatitis B who visited Hiroshima University Hospital between January 2000 to December 2009 who underwent liver biopsy for diagnosis of chronic hepatitis and agreed to provide blood samples for a viral hepatitis study were examined. Histological diagnosis was evaluated as described previously [Desmet et al., 1994]. Anti-HBs and anti-HBc antibodies were also examined in 22 healthy controls, all of whom tested negative for HBsAg and anti-HBc and anti-HCV antibodies. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consent for the study using a form approved by the ethical committee of Hiroshima University.

Viral Markers

Serum samples obtained at biopsy were kept frozen at $-80°C$ prior to analysis. Serum HBsAg and HBeAg levels were measured quantitatively using the Abbott Chemiluminescence Immunoassay Kit (Abbott Japan, Tokyo, Japan). HBV DNA levels were determined by the Cobas TaqMan HBV standardized real-time polymerase chain reaction (PCR) assay (Roche Molecular Systems, Pleasanton, CA). Results are expressed in log10 international units per milliliter.

MicroRNA Analysis

Circulating microRNA was extracted from 300 μl of serum samples using the mirVana PARIS Kit (Ambion,

TABLE I. Clinical Characteristics of Hepatitis B Virus Patients and Healthy Controls

	HBV patients (n = 198)		Healthy controls (n = 22)	
Characteristic	N	Value	N	Value
Age (years)[a]	198	42 (13–71)	22	31.5 (25–39)
Sex (male/female)	198	140/58	22	10/12
Fibrosis (1/2/3/4)	198	58/75/43/22		
Activity (0/1/2/3)	198	2/53/109/34		
miR-122/cel-miR-238	198	0.144 (0.002–1.737)	22	0.02 (0.01–0.04)
miR-22/cel-miR-238	198	0.266 (0.019–1.652)	22	0.02 (0.11–0.49)
HBV DNA (LGE/ml)[a]	181	6.5 (2.6–8.8)		
AST (IU/l)[a]	197	51 (18–982)		
ALT (IU/l)[a]	197	73 (10–1,867)	20	16 (10–23)
γ-GT (IU/l)[a]	189	46 (9–536)		
ALB (g/dl)[a]	196	4.3 (2.6–5.2)		
PLT ($\times 10^4$/mm^3)[a]	197	17.1 (1.0–36.2)		
PT[a]	180	92 (19–146)		
AFP (ng/ml)[a]	186	6.5 (<5.0–8,928.0)		
HBsAg (IU/ml)	176	2,765 (<0.05–1,55,000)		
Anti-HBeAg (±/NA)	176	104/82/12		
HBeAb (±/NA)	176	85/96/17		

NA, not available.
[a]Median (range).

Austin, TX) according to the manufacturer's instructions. RNA was eluted in 80 μl of nuclease free water and reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies Japan Ltd, Tokyo, Japan). *Caenorhabditis elegans* miR-238 (cel-miR-238) was spiked to each sample as a control for extraction and amplification steps. The reaction mixture contained 5 μl of RNA solution, 2 μl of 10× reverse transcription buffer, 0.2 μl of 100 mM dNTP mixture, 4 μl of 5× RT primer, 0.25 μl of RNase inhibitor, and 7.22 μl of nuclease free water in a total volume of 20 μl. The reaction was performed at 16°C for 30 min followed by 42°C for 30 min. The reaction was terminated by heating the solution at 85°C for 5 min. miR-122 and miR-22 were amplified using primers and probes provided by Applied Biosystems (Foster City, CA) using TaqMan MicroRNA assays according to the manufacturer's instructions. The reaction mixture contained 12.5 μl of 2× Universal PCR Master Mix, 1.25 μl of 20× TaqMan Assay solution, 1 μl of reverse

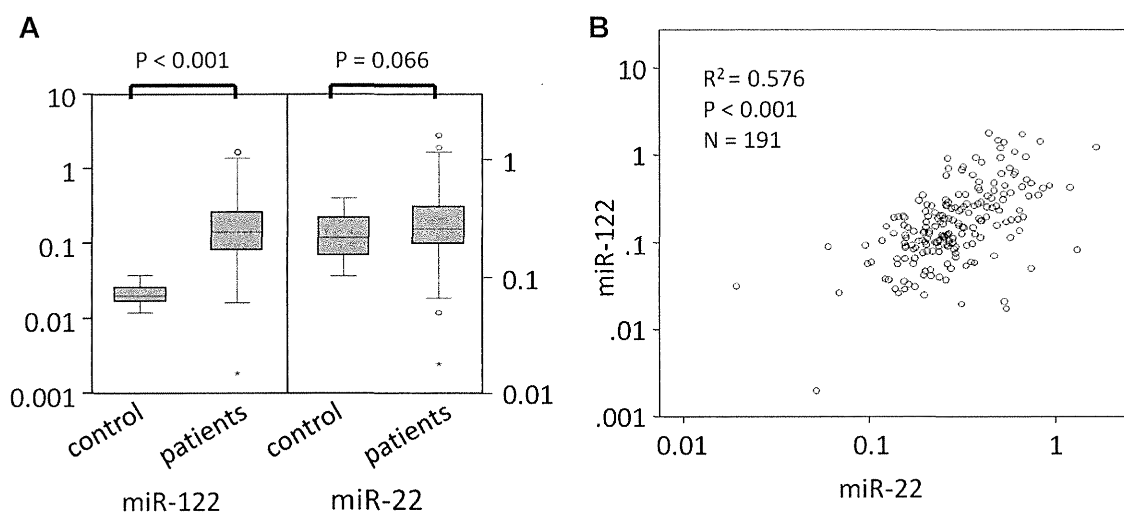

Fig. 1. Detection of miR-122 and miR-22 in patients infected with HBV and in healthy subjects and the relationship between miR-122 and miR-22. **A**: Serum levels of miR-122 and miR-22 in patients infected with HBV (171) and in healthy controls (22). Boxes represent 25–75 percentiles, and horizontal bars represent median values. Statistical analysis was performed using the Mann–Whitney U test. **B**: The relationship between miR-122 and miR-22 was analyzed using the Spearman rank correlation coefficient.

transcription product, and 10.25 μl of nuclease free water in a total volume of 25 μl. Amplification conditions were 95°C for 10 min followed by 50 denaturing cycles for 15 sec at 95°C and annealing and extension for 60 sec at 60°C in an ABI7300 thermal cycler. For the cel-miR-238 assay, a dilution series using chemically synthesized microRNA was used to generate a standard curve that permitted absolute quantification of molecules. For miR-122 and miR-22, relative abundance was determined using standard curves generated with a dilution series of samples with high serum levels. miR-122 and miR-22 levels were calculated by normalizing based on cel-miR-238 measurement levels.

Statistical Analysis

Data were analyzed using the Mann–Whitney U test for continuous variables and the chi-squared or Fisher exact test for categorical variables using the R statistics package (http://www.r-project.org). Factors associated with high miR-122 and miR-22 levels were analyzed by multiple regression analysis using the rms library. Forward/backward stepwise selection of factors with a P-value < 0.05 in univariate analysis was used for model selection. The Spearman rank correlation coefficient was used to evaluate the strength of the association between continuous variables.

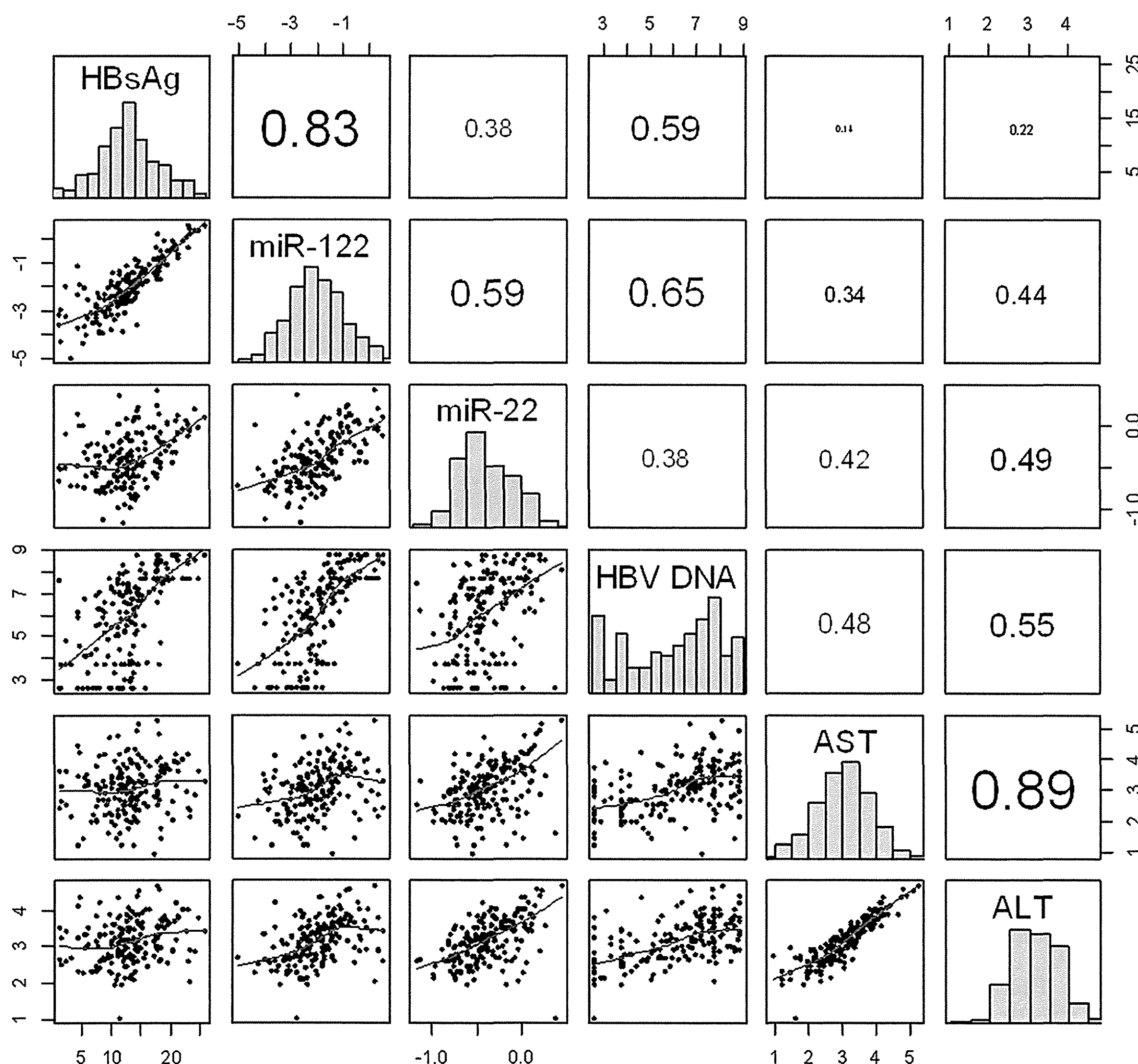

Fig. 2. Pairwise correlations of miR-122 and miR-22 with HBsAg, HBV DNA, ALT, and AST levels. Serum levels of miR-122 and miR-22 were compared with serum HBsAg and HBV DNA titers and with ALT and AST levels using the Spearman rank correlation coefficient.

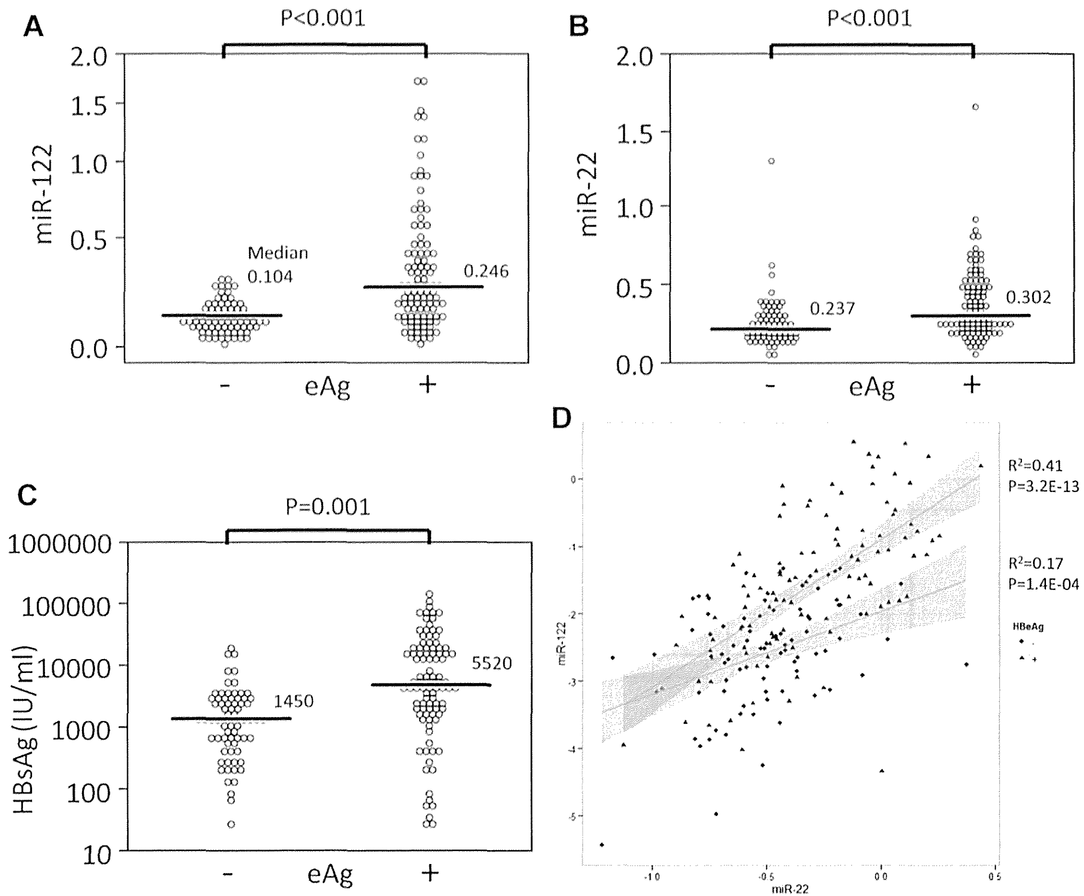

Fig. 3. Comparison of miR-122 and miR-22 with HBsAg levels between patients positive or negative for HBeAg. Serum levels of miR-122 (**A**), miR-22 (**B**), and HBsAg (**C**) were analyzed using the Mann–Whitney U test. Bars indicate median values.

RESULTS

Detection of Circulating miR-122 and miR-22 and Their Correlation

Both miR-122 and miR-22 were detectable in all HBV patients, and median values were higher than in normal controls (Table I; Fig. 1A, $P < 0.001$ and $P = 0.066$, respectively). miR-122 and miR-22 expression levels were moderately correlated (Fig. 1B, $R^2 = 0.576, P < 0.001$).

miR-122 and miR-22 Levels and Viral Markers

Relationships between miR-122 and miR-22 levels and HBsAg, HBeAg, and ALT levels were examined (Fig. 2A). There was a strong linear correlation between HBsAg and miR-122 levels ($R^2 = 0.824$, $P < 0.001$). There was also a correlation between HBsAg and miR-22 levels ($R^2 = 0.394$, $P < 0.001$), although the correlation was not as strong as with miR-122. Both miR-122 and miR-22 were also correlated with HBV DNA titers ($R^2 = 0.694$, $P < 0.001$

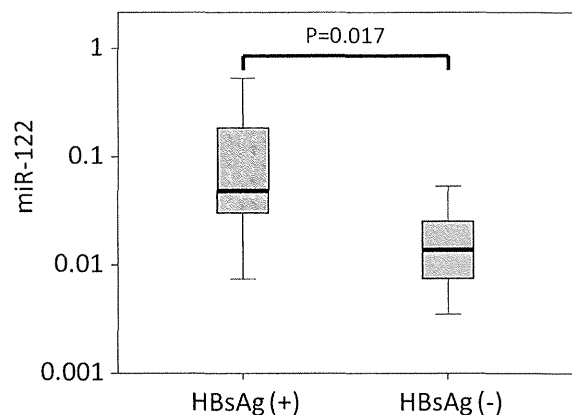

Fig. 4. miR-122 and HBsAg elimination. miR-122 levels before and after HBsAg elimination are shown for patients who became negative for HBsAg (n = 13). Bars represent median, minimum, and maximum levels, and boxes represent the 25th and 75th percentiles. Data were analyzed using the Mann–Whitney U test.

and $R^2 = 0.421$, $P < 0.001$, respectively) and ALT levels ($R^2 = 0.498$, $P < 0.001$ and $R^2 = 0.528$, $P < 0.001$, respectively). The correlation with ALT was slightly stronger with miR-22 ($R^2 = 0.528$) than with miR-122 ($R^2 = 0.498$). Patients who were positive for HBeAg had elevated levels of both miR-122 and miR-22 (Fig. 3A and B; $P < 0.001$ and $P < 0.001$) and had higher HBsAg titers (Fig. 3C; $P = 0.001$). The correlation between miR-122 and miR-22 expression was also stronger in HBeAg positive patients (Fig. 3D, $R^2 = 0.41$, $P = 3.2E-13$) compared to HBeAg negative patients ($R^2 = 0.17$, $P = 1.4E-04$).

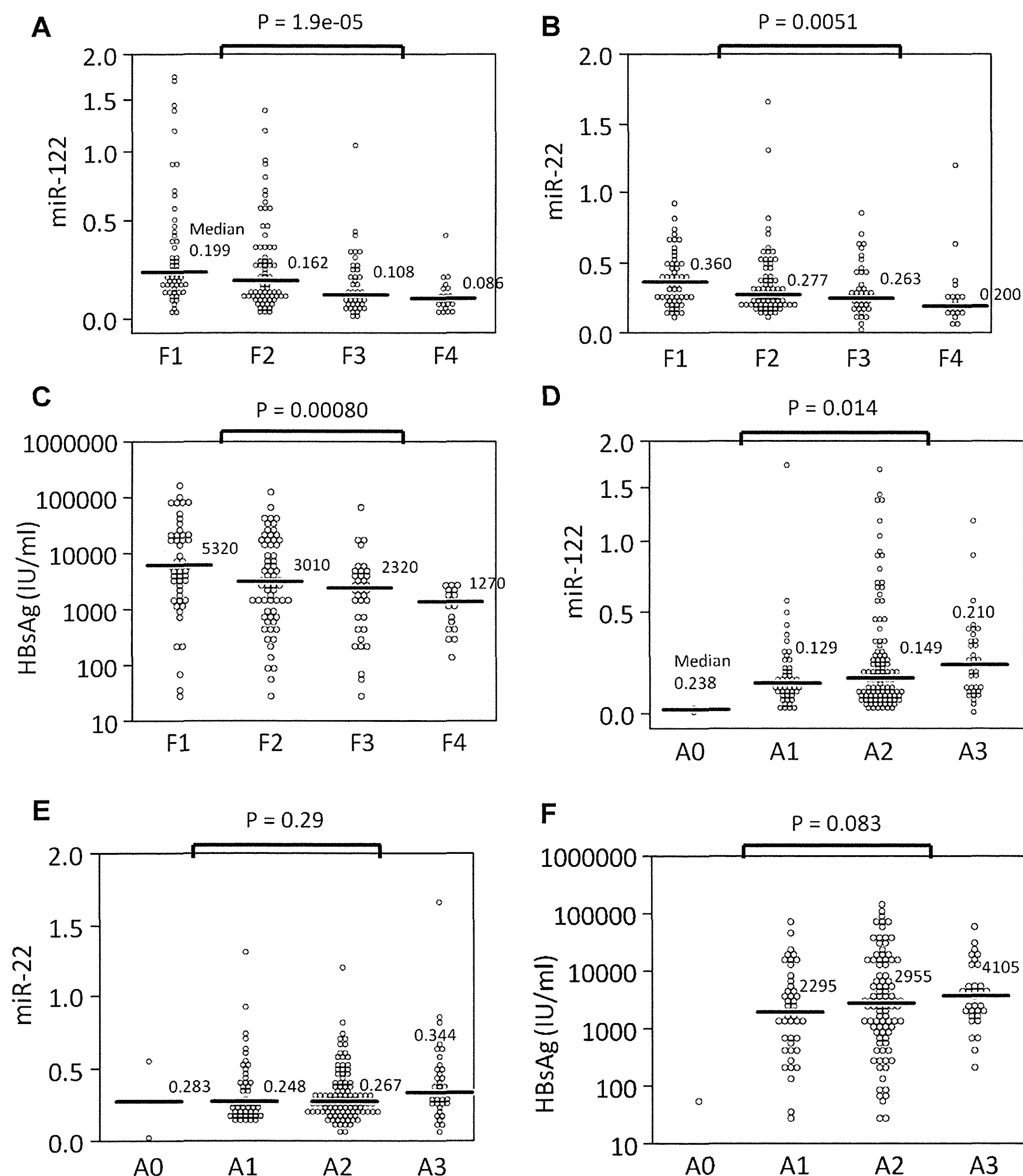

Fig. 5. Stage of fibrosis and histological inflammation activity by liver biopsy and miR-122, miR-22, and HBsAg levels. Serum levels of miR-122, miR-22, and HBsAg were plotted according to the stage of fibrosis (**A**, **B**, and **C**, respectively) and inflammation activity (**D**, **E**, and **F**). Median values are indicated as horizontal bars. Statistical analysis was performed using the Kruskal–Wallis non-parametric analysis of variance test.

miR-122 Levels in Patients Who Became Negative for HBsAg

To examine if the high miR-122 levels seen in chronic hepatitis B patients with high HBsAg levels result from active HBsAg production or represent individual characteristics that allow high-level HBsAg production, miR-122 levels were measured before and after elimination of HBsAg (observation period 4.5–16.5 years [median 9.0 years]). As shown in Figure 4, miR-122 levels in these patients declined significantly when they became negative for HBsAg ($P = 0.017$).

miR-122 and miR-22 Levels and Histological Findings

As shown in Figure 5A and B, both miR-122 and miR-22 were observed at progressively lower serum levels at more advanced stages of fibrosis ($P < 0.001$ and $P = 0.001$, respectively). HBsAg levels were also lower in patients with advanced fibrosis (Fig. 5C; $P = 0.001$). In contrast, serum levels of miR-122 and miR-22 were higher in patients with higher inflammatory activity (Fig. 5D and E; $P = 0.025$ and $P = 0.170$, respectively), although for miR-22 the difference was not significant. HBsAg levels were also marginally higher in patients with higher inflammatory activity (Fig. 5F; $P = 0.079$).

Factors Associated with Higher Serum miR-122 and miR-22 Levels

Clinical factors associated with elevated miR-122 and miR-22 levels were examined using multiple linear regression. As shown in Table II, HBsAg was most strongly associated with miR-122 ($P = 1.1E{-}67$), whereas serum AST levels were most strongly associated with miR-22 ($P = 4.7E{-}19$).

miR-122 and miR-22 Levels in Patients with Acute HBV Infection, Cirrhosis, and HCC

To examine miR-122 and miR-22 levels in patients with and without HBV infection, miR-122 and miR-22 levels were also measured in the following groups of patients: healthy controls (5), patients with acute (9) or chronic (9) HBV infection, liver cirrhosis (24),

TABLE II. Univariate and Multivariate Regression Analysis of Predictive Factors for MicroRNA-122 and MicroRNA-22 Expression Levels Relative to cel-miR-238

	Univariate				Multivariate	
MicroRNA	Variable	N	Coef.	*P*	Coef.	*P*
miR-122	Female	198	0.076	6.6E−01		
	Age	198	−0.030	2.9E−07***	0.007	1.7E−02*
	Fibrosis	198	−0.391	8.9E−07***	−0.143	3.8E−04***
	Activity	198	0.331	4.0E−03**		
	HBsAg	176	0.177	6.7E−46***	0.137	3.3E−32***
	HBeAg (±)	186	1.010	3.5E−11***		
	Anti-HBeAb (±)	181	−0.801	2.5E−07***		
	HBV DNA	181	0.357	2.1E−21***	0.064	1.4E−02*
	AST	197	0.472	6.1E−07***		
	ALT	197	0.816	2.0E−11***	0.281	4.1E−04***
	γ-GT	189	0.187	3.8E−01		
	Total bilirubin	196	−1.020	3.5E−02*	−0.596	9.8E−03**
	ALB	196	0.137	3.2E−02*		
	PT	180	0.020	1.3E−05***		
	AFP	186	0.000	1.5E−01		
	miR-22	198	2.010	1.9E−19***	0.739	4.2E−07***
miR-22	Female	198	−0.080	1.1E−01		
	Age	198	−0.009	3.8E−07***	−0.005	7.4E−04***
	Fibrosis	198	−0.085	2.9E−04***		
	Activity	198	0.053	1.1E−01		
	HBsAg	176	0.023	1.6E−07***	−0.016	2.1E−02*
	HBeAg (±)	186	0.192	1.8E−05***		
	Anti-HBeAb (±)	181	−0.143	1.5E−03**	−0.044	2.8E−01
	HBV DNA	181	0.058	4.6E−07***	−0.025	7.9E−02
	AST	197	0.161	1.5E−09***	0.116	2.6E−05***
	ALT	197	0.255	9.1E−14***		
	γ-GT	189	0.129	3.0E−02*		
	Total bilirubin	196	−0.170	2.2E−01		
	ALB	196	0.058	1.3E−03**		
	PT	180	0.006	1.6E−06***	0.004	2.3E−04***
	AFP	186	0.000	2.5E−01		
	miR-122	198	0.170	1.9E−19***	0.162	5.5E−06***

Forward/backward stepwise selection was used for model selection.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

HCV-related HCC (12), and HBV-related HCC (12). Both miR-122 and miR-22 were significantly elevated in patients with acute or chronic HBV infection compared to other case types (Fig. 6A and B) and were more strongly correlated (Fig. 6C).

DISCUSSION

In this study, expression levels of miR-122 and miR-22 were correlated with each other, as well as with markers of HBV infection, including HBsAg and HBV DNA titers (Fig. 2). Circulating levels of both microRNAs were also higher in patients who were positive for HBeAg. Although this suggests that these microRNAs may be up-regulated in cells infected with HBV, it will be necessary to compare serum and liver microRNA level to confirm this, as many other factors may influence circulating microRNA levels.

A notable result of this study is the strong linear association between miR-122 and serum HBsAg levels

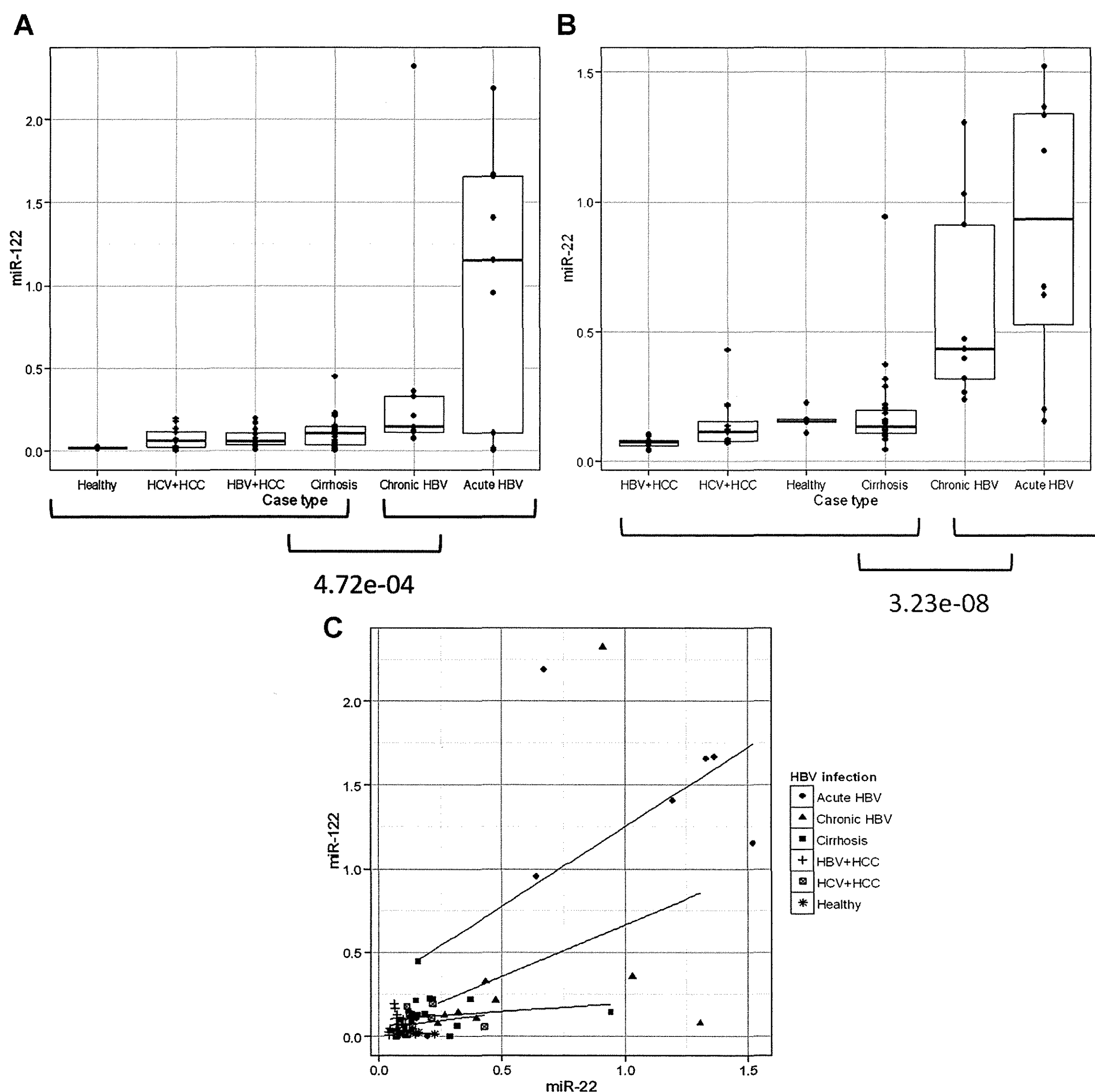

Fig. 6. miR-122 and miR-22 levels by case type. To examine the association of serum miR-122 and miR-22 with HBV infection, expression levels of miR-122 (**A**) and miR-22 (**B**) were compared among healthy controls, patients with acute or chronic HBV infection, liver cirrhosis, and HCC associated with either HBV or HCV. Both miR-122 and miR-22 were significantly higher in patients with acute or chronic HBV infection compared to patients with other case types, including patients with HBV-associated HCC. **C**: miR-122 and miR-22 also appear to be more strongly correlated in patients with acute or chronic HBV infection than in healthy controls or patients with cirrhosis or HCC.

(Table II; Fig. 2A). miR-122 has recently been shown to bind to a highly conserved HBV RNA sequence and negatively regulates viral gene expression and replication [Qiu et al., 2010; Chen et al., 2011]. Loss of miR-122 expression has also been shown to enhance HBV replication indirectly through cyclin G1-modulated p53 activity [Wang et al., 2011]. If miR-122 suppresses HBV replication, an inverse relationship between HBsAg titer and miR-122 levels might be expected, but instead a strong positive correlation was observed in this study. Although the reason for higher levels of miR-122 in patients with high HBsAg production is unclear, the innate immune response in liver cells against HBV replication may potentially induce higher expression of miR-122, which might be reflected in serum levels. Another possibility is that HBV might evade miR-122 suppression by sequestering and excreting miR-122 within the massively over-produced HBsAg particles in serum, in which case serum levels might be proportional to HBsAg levels but may not reflect miR-122 levels in the liver. It will be necessary to compare matched serum and liver miR-122 levels to address this issue.

In contrast to miR-122 levels, miR-22 expression was most strongly correlated with ALT and AST levels (Fig. 2; Table II). As it is known that miR-122 is expressed primarily or exclusively in hepatocytes [Mariana et al., 2002], the higher levels of miR-122 might reflect liver cell damage caused in the course of chronic hepatitis, and the same may be true for miR-22. Tissue-specificity of miR-22 is less clear, although it appears to be strongly expressed in hepatocellular carcinoma cell lines [Landgraf et al., 2007]. However, the fact that the levels of miR-22 are more strongly associated with ALT levels than miR-122 suggests that miR-122 is more likely to be over-expressed in liver cells infected with HBV. In this sense, miR-22 might be a better marker of liver injury than miR-122, although the lack of correlation of miR-22 with inflammatory activity complicates this association. Therefore, miR-122 and miR-22 may reflect different aspects of HBV infection and disease progression. miR-122 and miR-22 were expressed more strongly in acute and chronic HBV infection than in healthy controls or in patients with cirrhosis or HCC, suggesting an association with HBV infection, but notably miR-22 expression was comparatively higher in chronic HBV infection than miR-122 (Fig. 6). Measuring expression levels of one or both of these microRNAs may aid in assessment of disease severity [Waidmann et al., 2012].

In this study, miR-122 and miR-22 levels were both associated with HBV replication and liver injury. This suggests the need for a more systematic approach to examining multiple microRNAs under various chronic hepatitis B conditions and possibly in HBV-associated hepatocellular carcinoma. Further study is needed to establish a system to evaluate various disease conditions or prognoses in chronic HBV infection using microRNA biomarkers. It may also be of interest to determine the mechanism underlying the strong linear correlation between HBsAg and miR-122 levels to improve understanding of HBV virology.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleotide analogue treatments in chronic hepatitis B patients

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Abstract

Background Treatment for chronic hepatitis B has improved drastically with the use of nucleot(s)ide analogues (NAs). However, NA therapy typically fails to eliminate Hepatitis B virus (HBV) completely, and it is difficult to discontinue these therapies. We previously demonstrated that NA therapy induced immature viral particles, including HBV RNA in sera of chronic hepatitis B patients. In the study reported here, we analyzed the association between HBV RNA titer and the recurrence rate of hepatitis after discontinuation of NA therapy.

Methods The study cohort comprised 36 patients who had discontinued NA therapy. Serum HBV DNA or DNA plus RNA levels were measured by real time PCR and statistical analyses were performed using clinical data and HBV markers.

Results At 24 weeks after discontinuation of NA therapy, HBV DNA rebound was observed in 19 of the 36 patients (52.8 %), and alanine aminotransferase (ALT) rebound was observed in 12 of 36 patients (33.3 %). Multivariate statistical analysis was used to identify factors predictive of HBV DNA rebound. The HBV DNA + RNA titer following 3 months of treatment was significantly associated with HBV DNA rebound [$P = 0.043$, odds ratio (OR) 9.474, 95 % confidence interval (CI) 1.069–83.957)]. Absence of hepatitis B e antigen (HBeAg) at the end of treatment was significantly associated with ALT rebound ($P = 0.003$, OR 13.500, 95 % CI 2.473–73.705). In HBeAg-positive patients, the HBV DNA + RNA titer after 3 months of treatment was marginally associated with ALT rebound ($P = 0.050$, OR 8.032, 95 % CI 0.997–64.683).

Conclusions Monitoring of serum HBV DNA + RNA levels may be a useful method for predicting re-activation of chronic hepatitis B after discontinuation of NA therapy.

Keywords HBV · HBV RNA · Nucleotide analogue · HBV replication

Abbreviations

ADV Adefovir dipivoxil
ETV Entecavir

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HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
LMV	Lamivudine
NA	Nucleot(s)ide analogue
RT	Reverse transcriptase

Introduction

Hepatitis B virus (HBV) infection is a serious global health problem, with more than two billion people infected with HBV, of whom about 20 % remain chronically infected [1, 2]. Chronically infected individuals often develop chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), and the incidence of HCC in chronically infected individuals is significantly higher than that in healthy individuals [3]. Once HBV infects human hepatocytes, HBV genomes are transported into the nucleus, and some viral genomes become integrated into human chromosomes [4–7]. Thus, complete elimination of the virus is difficult, and patients are generally treated with interferon and nucleot(s)ide analogues (NAs) that suppress viral replication and prevent the progression of liver disease by combating inflammation [8–10]. However, long-term treatment with NAs is known to lead to the development of drug-resistant viral mutants, with the possible occurrence of a serious hepatitis flare-up (breakthrough hepatitis) [11–21]. To avoid the development of drug-resistant HBV, Japanese guidelines currently recommend that patients with chronic hepatitis B be treated with the eventual goal of reaching a "drug-free state" involving discontinuation of NAs [9]. However, there are at the present time no criteria for safely discontinuing NA therapy.

It has previously been reported that HBV particles, including particles of HBV RNA, are released from hepatocytes during NA treatment and become detectable in sera [22–25]. Commonly, in the course of HBV replication, pregenome RNAs are encapsidated into HBV core particles in the cytoplasm, and all pregenome RNAs are reverse transcribed into plus-stranded genomic DNA in the core particle [26]. However, during NA therapy, it is thought that NA strongly interferes with reverse transcription, causing excessive accumulation of HBV RNA particles in hepatocytes and leading to release without reverse transcription. In our previous study, we found that the existence of HBV RNA particles was significantly associated with the development of drug-resistant viruses [22]. This finding led us to consider that the existence of HBV RNA particles might be associated with HBV replication activity and that viruses with high replication activity produce high amounts of HBV RNA, leading to a greater opportunity for developing drug-resistance mutations. Therefore, we speculated that serum HBV RNA levels might be associated with HBV replication activity.

In the study reported here, several clinical parameters, including serum HBV DNA and HBV RNA titers, were analyzed with the aim of identifying factors predictive of the safe discontinuation of NA treatment. HBV replication activity and the deviation between serum HBV RNA and HBV DNA levels were found to be important predictors for the safe discontinuation of NA treatment.

Materials and methods

Patients

The study cohort comprised 36 Japanese chronic hepatitis B patients who had received NA therapy for more than 6 months at Hiroshima University Hospital or hospitals belonging to the Hiroshima Liver Study Group (http://home.hiroshima-u.ac.jp/naika1/research_profile/pdf/liver_study_group_e.pdf) and subsequently discontinued NA therapy. The discontinuation of NA therapy was decided at the discretion of the attending physicians, resulting in similar, but not uniform, criteria for discontinuation. In all analyses, the time of discontinuation was defined as the end of NA therapy. None of the patients were infected with other viruses, including human immunodeficiency virus or hepatitis C virus, and none had evidence of other liver diseases, such as auto-immune hepatitis or alcoholic liver disease. Patients with a total ethanol intake of >100 kg were excluded [27]. All patients gave written informed consent to participate in the study. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of Hiroshima University Hospital.

Baseline characteristics of the 36 patients are shown in Table 1. Thirty-one patients were treated with 100 mg/day of lamivudine (LMV), three were treated with 0.5 mg/day of entecavir (ETV), and two were treated with 10 mg/day of adefovir (ADV) monotherapy or LMV + ADV combination therapy. Twenty-six patients underwent sequential therapy, which included 6 months of conventional interferon therapy from 1 month prior to discontinuation until 5 months after discontinuation of NA therapy. Twenty-three patients were male and 13 were female. Median age at the onset of treatment was 43 years. Sixteen patients were positive for hepatitis e antigen (HBeAg). Blood samples were obtained from the patients before the beginning of therapy and every 4 weeks during the follow-up period. Biochemical and hematological tests were performed by the Hiroshima University Hospital laboratory.

The remaining sera were stored at −80 °C for further analysis.

Extraction and reverse transcription of HBV nucleic acid

Nucleic acid was extracted from 100 μL of serum by the SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μL of H_2O. Each extracted solution was divided into two aliquots. An 8.8-μL aliquot of the nucleic acid solutions was used for measuring HBV RNA. The solutions were reverse-transcribed as previously described [22]. The nucleic acid solutions were then mixed with 25 pM of random primer (Takara Bio, Shiga, Japan) and incubated at 65 °C for 5 min. The samples were set on ice for 5 min, then each sample was mixed with 4 μL of 5× reverse transcription (RT) buffer, 2 μL of 10 mM dNTPs, 2 μL of 0.1 M dithiothreitol (DTT), 8 U of ribonuclease inhibitor, and 100 U of M-MLV reverse transcriptase (ReverTra Ace; TOYOBO Co., Osaka, Japan). The reaction mixture was incubated at 30 °C for 10 min and 42 °C for 60 min, followed by inactivation at 99 °C for 5 min. The aliquots of the nucleic acid solutions were then used for the measurement of HBV DNA.

Table 1 Clinical backgrounds of the study cohort

Characteristics[a]	Values
Gender (M:F)	23:13
HBV genotype (B:C:ND)	2:31:3
Age (years)[b]	43 (25–66)
Platelet ($\times 10^4/\mu L$)[b]	16.1 (9.6–28.0)
ALT (IU/L)[b]	139 (22–780)
HBV DNA (log copies/mL)[b]	6.9 (3.6–8.8)
HBsAg (IU/mL)[b]	3,088 (66–1,354,400)
HBeAg (+:−)	16:20
HBcrAg (log U/mL)[b]	6.2 (3.4–8.8)
Nucleot(s)ide analogues (LMV:LMV + ADV:ADV:ETV)	31:1:1:3
Sequential therapy (+:−)	26:10
Duration of NA therapy (weeks)[b]	36 (24–304)
Observation period (weeks)[b]	269 (73–508)
Re-elevation of HBV DNA within 24 weeks (+:−)	21:15
Re-elevation of ALT within 24 weeks (+:−)	13:23

M Male, *F* female, *HBV* hepatitis B virus, *ND* not determined *ALT* alanine aminotransferase, *HBsAg* hepatitis B surface antigen, *HBeAg* hepatitis B e antigen, *HBcrAg* HBV core-related antigen, *LMV* lamivudine, *ADV* adefovir, *ETV* entecavir, *NA* nucleot(s)ide analogues

[a] Unless indicated otherwise, the values are given as the number (*n*) of patients

[b] Mean (range)

Measurement of serum HBV DNA and RNA by real-time PCR

The real-time PCR analyses were performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the instructions provided by the manufacturer. A 25-μL volume of reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of forward primer (5′-TTT GGGGCATGGACATTGAC-3′, nucleotides 1893–1912), 200 nM of reverse primer (5′-GGTGAACAATGGTCCG GAGAC-3′, nucleotides 2029–2049), and 1 μL of DNA or cDNA solution was prepared. After incubation for 2 min at 50 °C, the sample was heated for 10 min at 95 °C for denaturing, followed by a PCR cycling program consisting of 40 two-step cycles of 15 s each at 95 °C and 60 s at 60 °C. The lower detection limit of this assay was 2.3 log copies/mL. In the statistical analyses, samples which included less than the quantitation limit of HBV

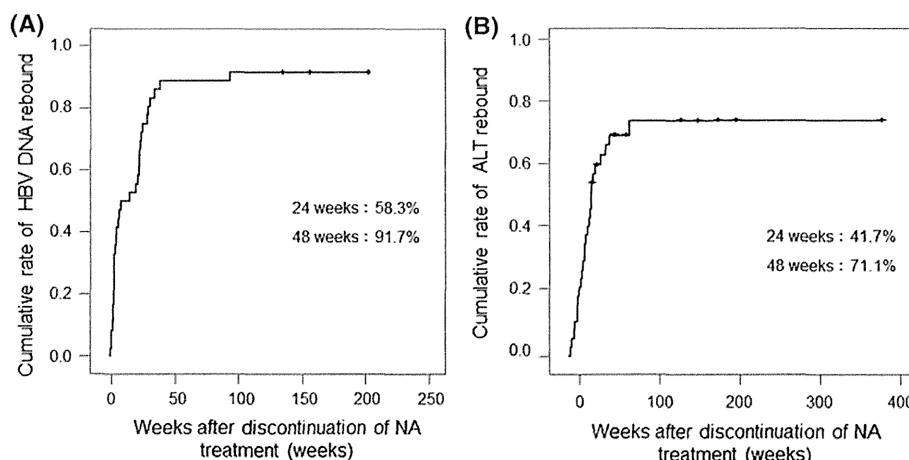

Fig. 1 Cumulative rate of hepatitis B virus (*HBV*) DNA (**a**) and alanine aminotransferase (*ALT*) rebound (**b**) in 36 chronic hepatitis B patients following discontinuation of nucleos(t)ide analogue (*NA*) therapy. Cumulative HBV DNA rebound rate and cumulative ALT rebound rate were analyzed using the Kaplan–Meier method

nucleotides were represented as 2.2 log copies/mL. By using these methods, we were able to measure the HBV DNA titers with DNA solutions and HBV DNA + RNA titers with cDNA solutions. In the present study, the ratios between HBV DNA + RNA to HBV DNA (DR ratio) was also assessed using the ratio of $\log_{10}$(HBV DNA + RNA) to $\log_{10}$(HBV DNA).

Measurement of HBV-related markers

Quantification of serum hepatitis B surface antigen (HBsAg) was performed with Elecsys HBsAg II Quant (Roche Diagnostics, Tokyo, Japan). High HBsAg titer was measured with 40,000-fold diluted serum. The quantitative range of HBsAg was 0.05–5,200,000 IU/mL. Serum HBcrAg levels were

Table 2 Multiple logistic regression for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment

Factors[a]	DNA relapsed (n = 21)	DNA non-relapsed (n = 15)	Univariate P value[b]	Multiple logistic regression[c]	
				P value	OR (95 % CI)
Gender (M:F)	12:9	11:4	0.484 (chi-square test)		
HBV genotype (B:C:ND)	1:18:2	1:13:1	0.931 (chi-square test)		
Before treatment					
Age (years)[d]	41 (25–59)	47 (30–66)	0.252		
Platelet ($\times 10^4/\mu$L)[d]	17.6 (9.6–28.0)	14.8 (9.6–23.6)	0.104		
ALT (IU/L)[d]	161 (37–780)	114 (22–304)	0.324		
HBsAg (IU/mL)[d]	3,714 (462–1,354,400)	1,754 (66–10,109)	0.083	0.581	
HBeAg (+:−)	12:9	4:11	0.096 (chi-square test)	0.389	
HBcrAg (log U/mL)[d]	5.9 (4.8–8.8)	6.2 (3.4–7.9)	0.608		
HBV DNA (log copies/mL)[d]	9.1 (3.5–10.1)	7.4 (4.1–9.3)	0.547		
HBV DNA + RNA titers (log copies/mL)	7.9 (3.4–10.0)	7.0 (3.4–9.1)	0.704		
DR ratio	−0.2 (−1.4–0.5)	−0.4 (−1.5 to 0.0)	0.304		
After 3 months of treatment					
HBV DNA (log copies/mL)[d]	4.4 (2.2–7.3)	3.6 (2.2–5.4)	0.056	0.074	
HBV DNA + RNA titers (log copies/mL)	4.8 (2.2–8.2)	4.2 (2.2–5.8)	0.015	0.043	9.474 (1.069–83.957)
DR ratio	0.9 (−0.9–2.7)	0.4 (−0.7 to 1.4)	0.019	0.643	
End of treatment					
HBsAg (IU/mL)[d]	1,912 (481–16,301)	470 (<1.1–4,736)	0.036	0.070	
HBeAg (+:−)	11:10	3:12	0.083 (chi-square test)	0.637	
HBcrAg (log U/mL)[d]	4.9 (3.0–8.2)	4.2 (3.0–6.6)	0.516		
HBV DNA (log copies/mL)[d]	3.5 (2.2–9.2)	3.3 (2.2–7.1)	0.465		
HBV DNA + RNA titers (log copies/mL)	3.9 (2.2–8.7)	3.6 (2.2–6.5)	0.117		
DR ratio	0.7 (−1.0–2.7)	0.0 (−1.0 to 1.2)	0.102		
Sequential therapy (+:−)	13:8	13:2	0.142 (chi-square test)		
Duration of treatment (weeks)[d]	34 (24–221)	53 (24–304)	0.800		

DR ratio HBV DNA + RNA titers/HBV DNA, *OR* odds ratio, *CI* confidence interval

[a] Unless indicated otherwise, the values are given as the number (n) of patients

[b] Univariate analysis was performed with Mann-Whitney U test unless indicated otherwise

[c] Multiple logistic regression analysis was performed using variables that were at least marginally significant ($P < 0.10$) in the univariate analysis

[d] Median (range)