Biochemical and Biophysical Research Communications 441 (2013) 230–235

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Keiichi Kosaka [a,b], Nobuhiko Hiraga [a,b], Michio Imamura [a,b], Satoshi Yoshimi [a,b], Eisuke Murakami [a,b], Takashi Nakahara [a,b], Yoji Honda [a,b], Atsushi Ono [a,b], Tomokazu Kawaoka [a,b], Masataka Tsuge [a,b], Hiromi Abe [a,b], C. Nelson Hayes [a,b,c], Daiki Miki [b,c], Hiroshi Aikata [a,b], Hidenori Ochi [b,c], Yuji Ishida [b,d], Chise Tateno [b,d], Katsutoshi Yoshizato [b,d], Tamito Sasaki [a,b], Kazuaki Chayama [a,b,c,*]

[a] Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Science, Hiroshima University, Hiroshima, Japan
[b] Liver Research Project Center, Hiroshima University, Hiroshima, Japan
[c] Laboratory for Digestive Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Hiroshima, Japan
[d] PhoenixBio Co., Ltd., Higashihiroshima, Japan

ARTICLE INFO

*Article history:*
Received 20 September 2013
Available online 16 October 2013

*Keywords:*
Human hepatocyte chimeric mouse
TK-NOG mouse
uPA–SCID mouse
Hepatitis B virus
Hepatitis C virus
Human serum albumin

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.



## 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.

* Corresponding author. Address: Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Fax: +81 82 255 6220.
*E-mail address:* chayama@hiroshima-u.ac.jp (K. Chayama).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.10.040

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

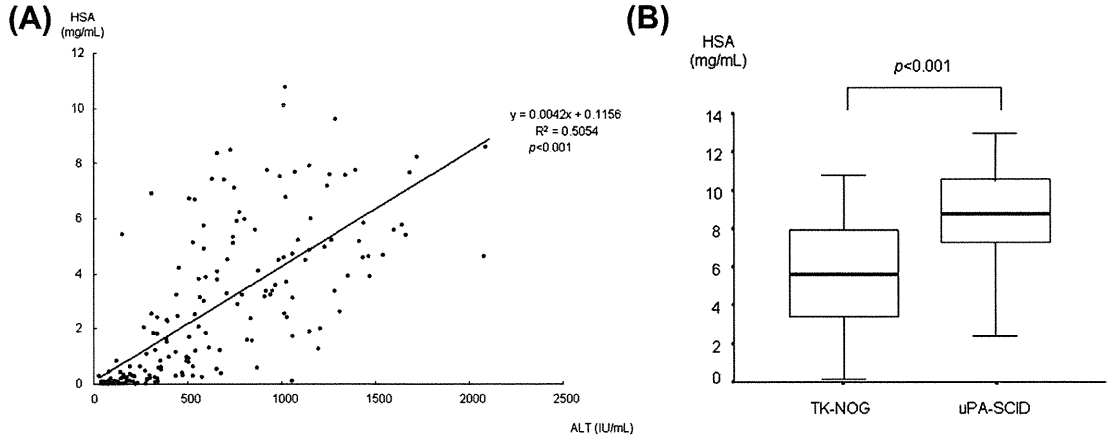


**Fig. 1.** Human hepatocyte repopulation index in humanized mice. Serum alaninaminotransferase (ALT) levels in TK-NOG mice were measured one week after ganciclovir treatment. Human serum albumin (HSA) levels were measured eight weeks after transplantation of human hepatocytes. (A) Correlation between 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

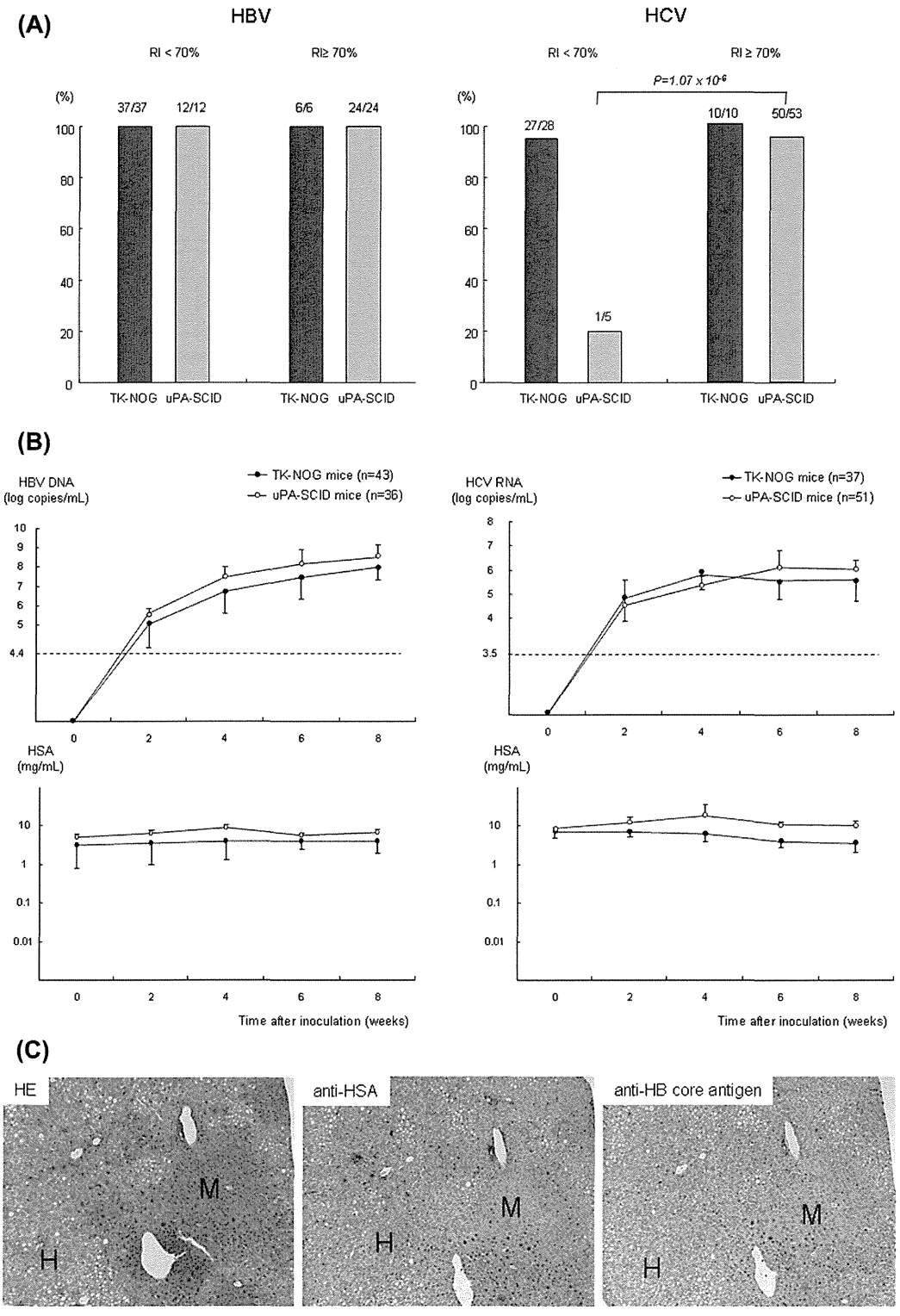


**Fig. 2.** Hepatitis viruses infection in chimeric mice. (A) Eight weeks after human hepatocyte transplantation, mice with serum HSA level over 1 mg/mL were inoculated with HBV- or HCV-positive human serum samples. Percentages of mice that became positive for HBV DNA (left panel) or HCV RNA (right panel) 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

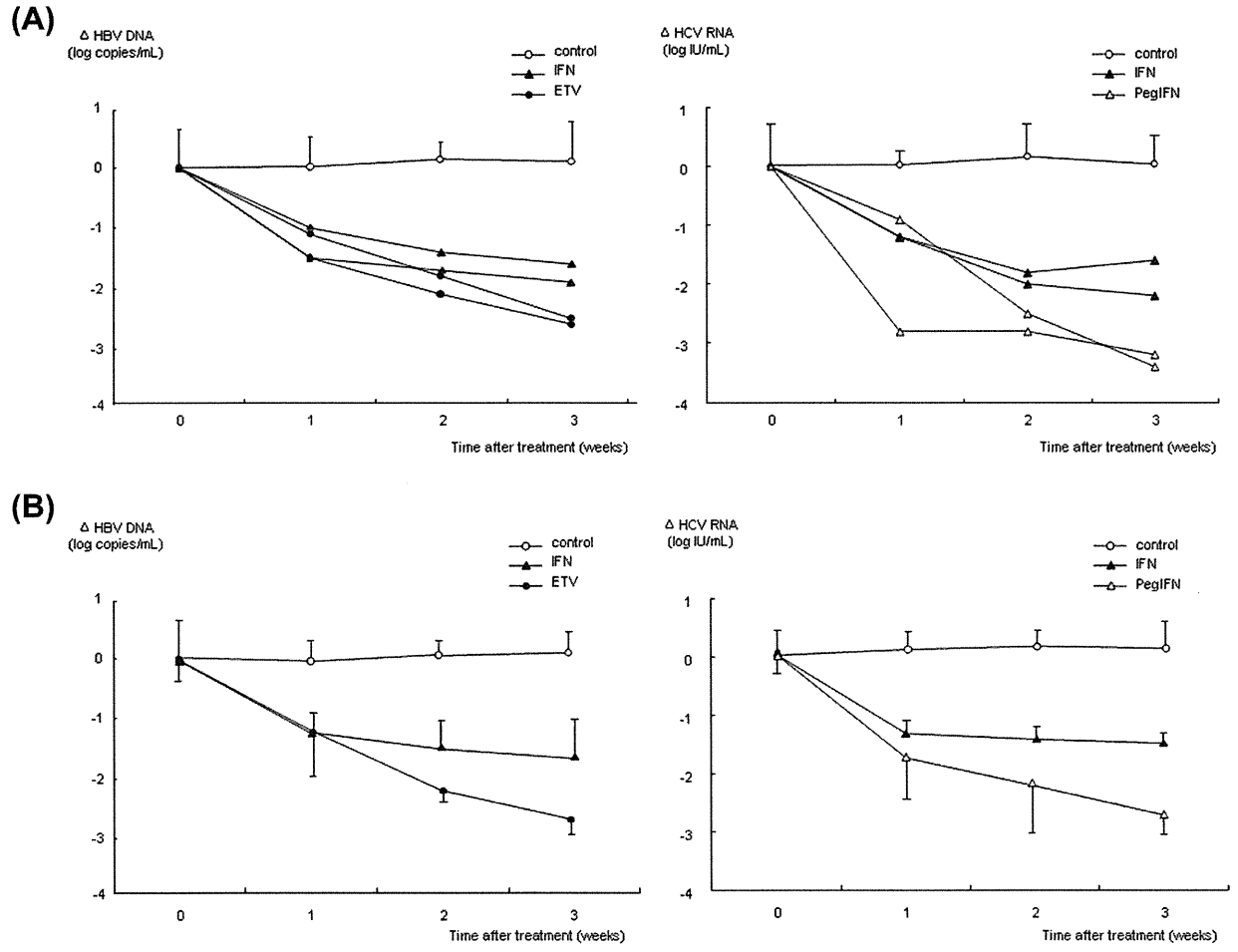


**Fig. 3.** Reduction of serum viral titers in mice treated with anti-viral agents. (A) HBV- (left panel) or HCV-infected (right panel) TK-NOG mice were treated with entecavir, interferon (IFN)-alpha or PegIFN-alpha-2a. Control: HBV- and HCV-infected mice without antiviral treatment. (B) HBV- (left panel) 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 ($n = 4$).

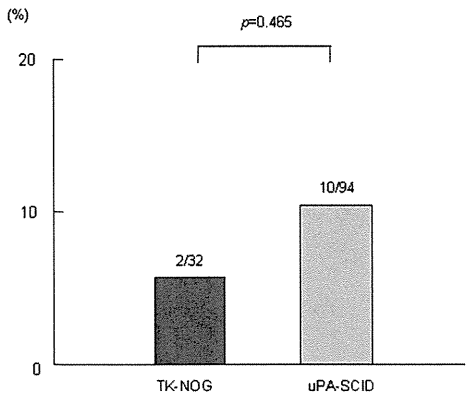


**Fig. 4.** Frequency of unexpected death within 8 weeks in mice. The numbers of sudden deaths occurring within 8 weeks of viral infection in TK-NOG mice and uPA–SCID mice are shown as bars.

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 antiviral 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.

## Financial support

This work was supported by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor and Welfare and Ministry of Education Culture Sports Science and Technology, Government of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

## Acknowledgments

The authors thank Rie Akiyama, and Yoko Matsumoto for their expert technical help. This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor, Health and Welfare.

## References


[1] W.C. Maddrey, Hepatitis B: an important public health issue, J. Med. Virol. 61 (2000) 362–366.

[2] Global surveillance and control of hepatitis C, Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium, J. Viral Hepat. 6 (1999) 35–47.

[3] R.P. Beasley, Hepatitis B virus. The major etiology of hepatocellular carcinoma, Cancer 61 (1988) 1942–1956.

[4] D.F. Mercer, D.E. Schiller, J.F. Elliott, D.N. Douglas, C. Hao, A. Rinfret, W.R. Addison, K.P. Fischer, T.A. Churchill, J.R. Lakey, D.L. Tyrrell, N.M. Kneteman, Hepatitis C virus replication in mice with chimeric human livers, Nat. Med. 7 (2001) 927–933.

[5] C. Tateno, Y. Yoshizane, N. Saito, M. Kataoka, R. Utoh, C. Yamasaki, A. Tachibana, Y. Soeno, K. Asahina, H. Hino, T. Asahara, T. Yokoi, T. Furukawa, K. Yoshizato, Near completely humanized liver in mice shows human-type metabolic responses to drugs, Am. J. Pathol. 165 (2004) 901–912.

[6] H. Azuma, N. Paulk, A. Ranade, C. Dorrell, M. Al-Dhalimy, E. Ellis, S. Strom, M.A. Kay, M. Finegold, M. Grompe, Robust expansion of human hepatocytes in Fah-/-/Rag2-/-/Il2rg-/-mice, Nat. Biotechnol. 25 (2007) 903–910.

[7] K.D. Bissig, T.T. Le, N.B. Woods, I.M. Verma, Repopulation of adult and neonatal mice with human hepatocytes: a chimeric animal model, Proc. Natl. Acad. Sci. USA 104 (2007) 20507–20511.

[8] H. Suemizu, M. Hasegawa, K. Kawai, K. Taniguchi, M. Monnai, M. Wakui, M. Suematsu, M. Ito, G. Peltz, M. Nakamura, Establishment of a humanized model of liver using NOD/Shi-scid IL2Rgnull mice, Biochem. Biophys. Res. Commun. 377 (2008) 248–252.

[9] Y.P. de Jong, C.M. Rice, A. Ploss, New horizons for studying human hepatotropic infections, J. Clin. Invest. 120 (2010) 650–653.

[10] Z. He, H. Zhang, X. Zhang, D. Xie, Y. Chen, K.J. Wangensteen, S.C. Ekker, M. Firpo, C. Liu, D. Xiang, X. Zi, L. Hui, G. Yang, X. Ding, Y. Hu, X. Wang, Liver xeno-repopulation with human hepatocytes in Fah-/-Rag2-/-mice after pharmacological immunosuppression, Am. J. Pathol. 177 (2010) 1311–1319.

[11] K.D. Bissig, S.F. Wieland, P. Tran, M. Isogawa, T.T. Le, F.V. Chisari, I.M. Verma, Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment, J. Clin. Invest. 120 (2010) 924–930.

[12] M. Tsuge, N. Hiraga, H. Takaishi, C. Noguchi, H. Oga, M. Imamura, S. Takahashi, E. Iwao, Y. Fujimoto, H. Ochi, K. Chayama, C. Tateno, K. Yoshizato, Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus, Hepatology 42 (2005) 1046–1054.

[13] E. Ohara, N. Hiraga, M. Imamura, E. Iwao, N. Kamiya, I. Yamada, T. Kono, M. Onishi, D. Hirata, F. Mitsui, T. Kawaoka, M. Tsuge, S. Takahashi, H. Abe, C.N. Hayes, H. Ochi, C. Tateno, K. Yoshizato, S. Tanaka, K. Chayama, Elimination of hepatitis C virus by short term NS3–4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice, J. Hepatol. 54 (2011) 872–878.

[14] N. Hiraga, H. Abe, M. Imamura, M. Tsuge, S. Takahashi, C.N. Hayes, H. Ochi, C. Tateno, K. Yoshizato, Y. Nakamura, N. Kamatani, K. Chayama, Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus, Hepatology 54 (2011) 764–771.

[15] M. Hasegawa, K. Kawai, T. Mitsui, K. Taniguchi, M. Monnai, M. Wakui, M. Ito, M. Suematsu, G. Peltz, M. Nakamura, H. Suemizu, The reconstituted 'humanized liver' in TK-NOG mice is mature and functional, Biochem. Biophys. Res. Commun. 405 (2011) 405–410.

[16] H. Yamazaki, H. Suemizu, N. Murayama, M. Utoh, N. Shibata, M. Nakamura, F.P. Guengerich, In vivo drug interactions of the teratogen thalidomide with midazolam: heterotropic cooperativity of human cytochrome P450 in humanized TK-NOG mice, Chem. Res. Toxicol. 26 (2013) 486–489.

[17] H. Yamazaki, H. Suemizu, M. Shimizu, S. Igaya, N. Shibata, M. Nakamura, G. Chowdhury, F.P. Guengerich, In vivo formation of dihydroxylated and glutathione conjugate metabolites derived from thalidomide and 5-Hydroxythalidomide in humanized TK-NOG mice, Chem. Res. Toxicol. 25 (2012) 274–276.

[18] Y. Hu, M. Wu, T. Nishimura, M. Zheng, G. Peltz, Human pharmacogenetic analysis in chimeric mice with 'humanized livers', Pharmacogenet. Genomics 23 (2013) 78–83.

[19] N. Hiraga, M. Imamura, M. Tsuge, C. Noguchi, S. Takahashi, E. Iwao, Y. Fujimoto, H. Abe, T. Maekawa, H. Ochi, C. Tateno, K. Yoshizato, A. Sakai, Y. Sakai, M. Honda, S. Kaneko, T. Wakita, K. Chayama, Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon, FEBS Lett. 581 (2007) 1983–1987.

[20] T. Vanwolleghem, L. Libbrecht, B.E. Hansen, I. Desombere, T. Roskams, P. Meuleman, G. Leroux-Roels, Factors determining successful engraftment of hepatocytes and susceptibility to hepatitis B and C virus infection in uPA-SCID mice, J. Hepatol. 53 (2010) 468–476.

[21] M. Ito, H. Hiramatsu, K. Kobayashi, K. Suzue, M. Kawahata, K. Hioki, Y. Ueyama, Y. Koyanagi, K. Sugamura, K. Tsuji, T. Heike, T. Nakahata, NOD/SCID/gamma(c) (null) mouse: an excellent recipient mouse model for engraftment of human cells, Blood 100 (2002) 3175–3182.

[22] P. Meuleman, L. Libbrecht, R. De Vos, B. de Hemptinne, K. Gevaert, J. Vandekerckhove, T. Roskams, G. Leroux-Roels, Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera, Hepatology 41 (2005) 847–856.

[23] X. Wang, H. Willenbring, Y. Akkari, Y. Torimaru, M. Foster, M. Al-Dhalimy, E. Lagasse, M. Finegold, S. Olson, M. Grompe, Cell fusion is the principal source of bone-marrow-derived hepatocytes, Nature 422 (2003) 897–901.

J Gastroenterol (2013) 48:1188–1204
DOI 10.1007/s00535-012-0737-2

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

**Masataka Tsuge · Eisuke Murakami · Michio Imamura · Hiromi Abe · Daiki Miki · Nobuhiko Hiraga · Shoichi Takahashi · Hidenori Ochi · C. Nelson Hayes · Hiroyuki Ginba · Kazuhiro Matsuyama · Hiroiku Kawakami · Kazuaki Chayama**

Received: 29 August 2012 / Accepted: 12 December 2012 / Published online: 9 February 2013


**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

M. Tsuge · E. Murakami · M. Imamura · H. Abe · D. Miki · N. Hiraga · S. Takahashi · H. Ochi · C. Nelson Hayes · K. Chayama
Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

M. Tsuge · E. Murakami · M. Imamura · H. Abe · D. Miki · N. Hiraga · S. Takahashi · H. Ochi · C. Nelson Hayes · K. Chayama
Liver Research Project Center, Hiroshima University, Hiroshima, Japan

M. Tsuge
Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan

H. Ginba · K. Matsuyama
Virology/Microbiology Group, Molecular Diagnostics Marketing Department, Marketing Division, In Vitro Diagnostics Business Unit, Roche Diagnostics K.K, Tokyo, Japan

H. Kawakami
Kawakami Clinic, Hiroshima, Japan

K. Chayama (✉)
Division of Frontier Medical Science, Department of Medical and Molecular Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, Hiroshima 734-8551, Japan
e-mail: chayama@hiroshima-u.ac.jp

| | |
|---|---|
| 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

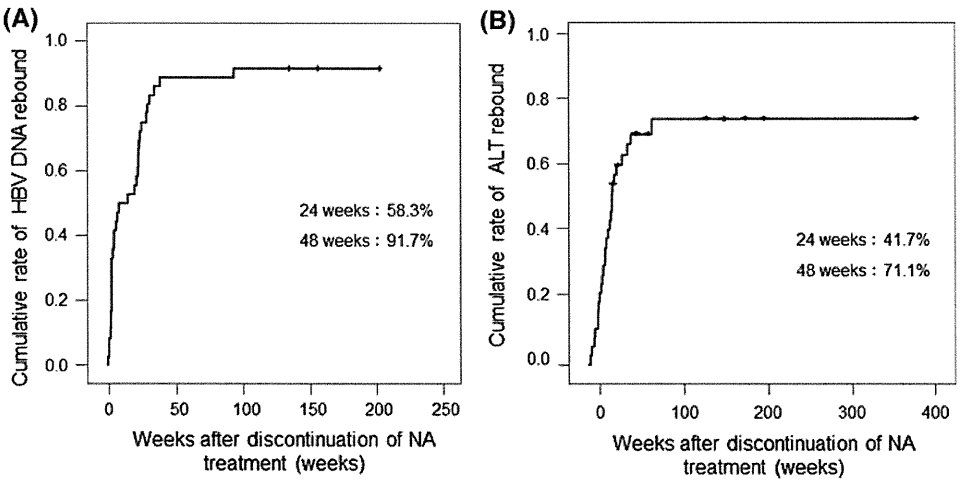


**Fig. 1** Cumulative rate of hepatitis B virus (*HBV*) DNA (**a**) and alanine aminotransferase (*ALT*) rebound (**b**) in 36 chronic hepatitis B patients following discontinuation of nucleos(t)ide analogue (*NA*) therapy. Cumulative HBV DNA rebound rate and cumulative ALT rebound rate were analyzed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

**Table 3** Univariate analysis for factors associated with HBV DNA rebound within 48 weeks after discontinuation of NA treatment

| Factors | DNA relapsed ($n = 31$) | DNA non-relapsed ($n = 5$) | Univariate $P$ value |
|---|---|---|---|
| Gender (M:F) | 21:10 | 2:3 | 0.328[b] |
| HBV genotype (B:C:ND) | 2:27:2 | 0:4:0 | 0.523[b] |
| Before treatment | | | |
| Age (years)[a] | 41 (25–66) | 47 (30–62) | 0.749 |
| Platelet ($\times 10^4/\mu L$)[a] | 15.6 (9.6–28.0) | 17.3 (14.7–18.8) | 0.679 |
| ALT (IU/L)[a] | 135 (22–780) | 192 (94–296) | 0.450 |
| HBsAg (IU/mL)[a] | 2,983 (66–1,354,400) | 4,264 (1,172–10,109) | 0.758 |
| HBeAg (+:−) | 14:17 | 2:3 | 1.000 |
| HBcrAg (log U/mL)[a] | 5.4 (3.4–8.8) | 6.8 (5.4–7.9) | 0.330 |
| HBV DNA (log copies/mL)[a] | 7.6 (3.5–10.1) | 8.3 (6.7–9.1) | 0.766 |
| HBV DNA + RNA titers (log copies/mL) | 7.4 (3.4–10.0) | 8.0 (6.7–9.0) | 0.522 |
| DR ratio | −0.2 (−1.4–0.9) | −0.3 (−0.6 to −0.1) | 0.596 |
| After 3 months of treatment | | | |
| HBV DNA (log copies/mL)[a] | 4.0 (2.2–7.3) | 3.7 (3.2–4.2) | 0.409 |
| HBV DNA + RNA titers (log copies/mL) | 4.8 (2.2–8.2) | 4.3 (2.7–4.9) | 0.507 |
| DR ratio | 0.7 (−0.9–2.7) | 0.6 (−0.6–1.4) | 0.464 |
| End of treatment | | | |
| HBsAg (IU/mL)[a] | 2,195 (48–16,301) | 533 (<1.1–9,680) | 0.105 |
| HBeAg (+:−) | 13:18 | 1:4 | 0.628[b] |
| HBcrAg (log U/mL)[a] | 4.7 (3.0–8.2) | 4.6 (3.6–6.6) | 0.657 |
| HBV DNA (log copies/mL)[a] | 3.5 (2.1–9.2) | 3.0 (2.7–6.1) | 0.818 |
| HBV DNA + RNA titers (log copies/mL) | 3.7 (2.2–8.7) | 4.2 (2.2–5.7) | 0.801 |
| DR ratio | 0.2 (−1.0–2.7) | 0.4 (−0.8–1.2) | 0.348 |
| Sequential therapy (+:−) | 23:8 | 3:2 | 0.603[b] |
| Duration of treatment (weeks)[a] | 36 (24–221) | 86 (24–304) | 0.278 |

*ND* not determined, *DR ratio* HBV DNA + RNA titers/HBV DNA

[a] Median (range) univariate analysis was performed with Mann-Whitney $U$ test

[b] Chi-square test

measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc, Tokyo, Japan), as described previously [28, 29].

## Evaluation of rebound of HBV DNA and alanine aminotransferase after discontinuation of NA therapy

The rebound of HBV DNA after discontinuation of NA therapy was determined based on two criteria: (1) when the HBV DNA reached >4.0 log copies/mL after discontinuation of NA therapy in patients whose HBV DNA titers became negative (<2.6 log copies/mL) at the end of NA therapy; (2) when the HBV DNA increased to >1.0 log copies/mL after the discontinuation of NA therapy in patients whose HBV DNA titers were still positive (>2.7 log copies/mL) at the end of NA therapy.

Alanine aminotransferase (ALT) rebound after discontinuation of NA therapy was defined using the following criteria: (1) when ALT reached >50 IU/L after discontinuation of NA therapy in those patients whose ALT levels had normalized (≤35 IU/L) at the end of NA therapy; (2) when ALT increased by >80 IU/L (twofold of upper limit of normal) after discontinuation of NA therapy in those patients whose ALT levels were still high (>35 IU/L) at the end of NA therapy.

## Statistical analysis

The baseline characteristics of the patients in the two groups were compared, and differences were assessed by the chi-square test with Yate's correction, Fisher's exact probability test, and the Mann–Whitney $U$ test. All $P$ values of <0.05 by the two-tailed test were considered to be significant. To identify predictors for HBV DNA or ALT rebound, univariate and multivariate logistic regression analyses were performed. Potential predictive factors included the following variables: age, gender, body mass index (BMI), platelet count, prothrombin time, total

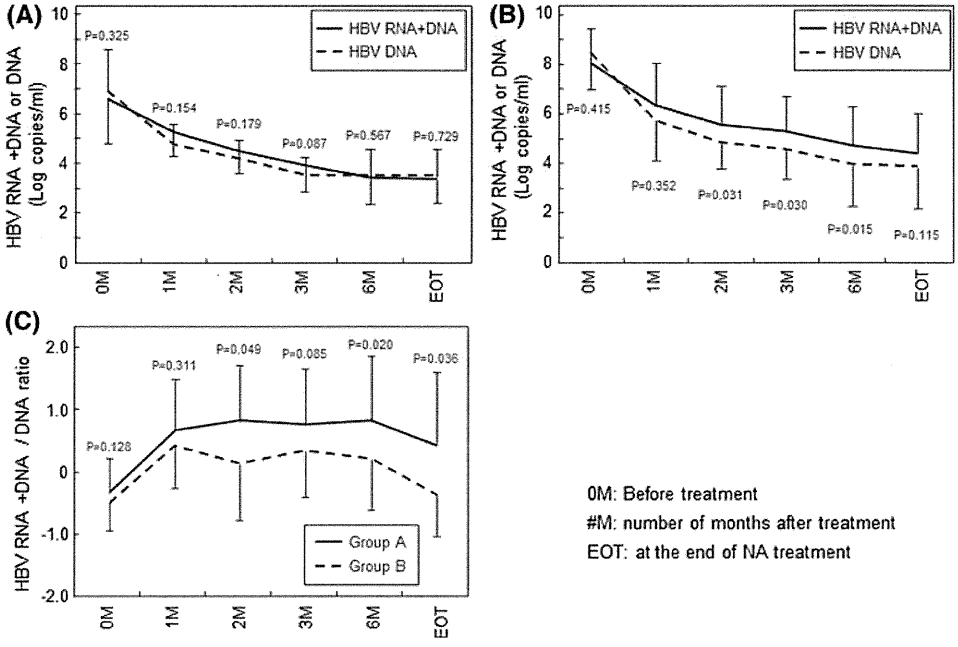


**Fig. 2** Change in HBV DNA and HBV DNA + RNA titers during NA therapy. **a**, **b** HBV DNA + RNA titers and HBV DNA titers were compared at each time point for the DNA non-relapse group (**a**) and DNA relapse group (**b**). **c** Changes in the HBV RNA + DNA/HBV DNA ratio were compared with each group. Statistical analyses were performed by the Mann–Whitney *U* test

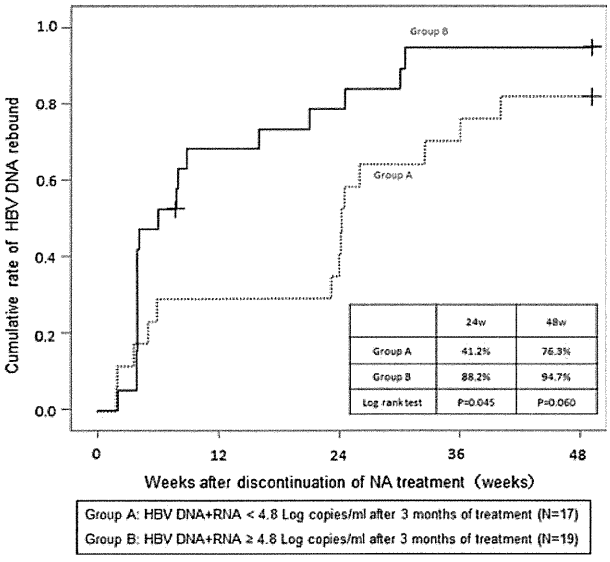


**Fig. 3** Cumulative rate of HBV DNA rebound after discontinuation of NA treatment. Seventeen patients whose HBV DNA + RNA titers reached <4.8 log copies/mL after 3 months of treatment, were assigned to group A; the other 19 patients, whose HBV DNA + RNA titers were ≥4.8 log copies/mL after 3 months of treatment, were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

bilirubin, aspartate aminotransferase, ALT, lactate dehydrogenase, alkaline phosphatase, gamma-glutamyltranspeptidase, HBV DNA titer, HBV DNA + RNA titer, and the DR ratio. As shown in a previous study, interferon treatment decreases the production of HBV RNA particles [23]. Thus, HBV RNA + DNA titer at 6 months of treatment was considered to be inappropriate for the statistical analyses in the present study, and these data were not included in these analyses. Odds ratios (OR) and 95 % confidence intervals (95 % CI) were also calculated. Variables with at least marginal significance ($P < 0.10$) in the univariate analysis were entered into the multiple logistic regression analysis to identify significant independent factors. Statistical analyses were performed using SPSS ver. 17.0 (SPSS, Chicago, IL).

## Results

### Analysis of HBV DNA and ALT rebound rates after discontinuation of NA therapy

Although NA therapy suppressed HBV replication and genomic HBV DNA synthesis, serum HBV DNA and ALT rebound occurred with a high frequency after therapy discontinuation. The cumulative HBV DNA and ALT rebound rates were analyzed to identify associated risk factors. As shown in Fig. 1a, the cumulative HBV DNA rebound rate increased in a time-dependent manner, reaching 58.3 and 91.7 % at 24 and 48 weeks after discontinuation of NA therapy, respectively. The cumulative

**Table 4** Univariate analysis for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment in those patients whose HBV DNA titer became negative at the end of NA treatment

| Factors[a] | DNA relapsed ($n = 5$) | DNA non-relapsed ($n = 6$) | Univariate $P$ value[b] |
|---|---|---|---|
| Gender (M:F) | 3:2 | 4:1 | 0.545 (chi-square test) |
| HBV genotype (B:C:ND) | 0:4:1 | 0:6:0 | 0.455 (chi-square test) |
| Before treatment | | | |
| Age (years)[c] | 41 (3–52) | 54 (32–66) | 0.119 |
| Platelet ($\times 10^4/\mu L$)[c] | 18.8 (11.7–27.5) | 14.8 (10.2–23.6) | 0.221 |
| ALT (IU/L)[c] | 186 (79–303) | 95 (48–270) | 0.273 |
| HBsAg (IU/mL)[c] | 2,603 (2,064–9,400) | 1,984 (406–7,016) | 0.180 |
| HBeAg (+:−) | 2:3 | 1:5 | 0.545 (chi-square test) |
| HBcrAg (log U/mL)[c] | 5.4 (5.0–7.8) | 4.1 (3.4–7.9) | 0.462 |
| HBV DNA (log copies/mL)[c] | 5.7 (3.8–9.2) | 7.9 (5.7–9.7) | 0.410 |
| HBV DNA + RNA titers (log copies/mL) | 5.6 (3.4–9.0) | 7.5 (5.0–9.7) | 0.583 |
| DR ratio | −0.1 (−0.8–0.1) | −0.4 (−0.7–0.0) | 0.527 |
| After 3 months of treatment | | | |
| HBV DNA (log copies/mL)[c] | 3.8 (2.2–4.8) | 3.5 (2.2–4.4) | 0.518 |
| HBV DNA + RNA titers (log copies/mL) | 4.0 (3.7–6.0) | 3.6 (2.2–4.8) | 0.313 |
| DR ratio | 1.2 (−0.1 to 1.4) | 0.4 (−0.9 to 0.7) | 0.272 |
| End of treatment | | | |
| HBsAg (IU/mL)[c] | 5,681 (684–16,301) | 1,865 (85–5,711) | 0.144 |
| HBeAg (+:−) | 1:4 | 1:5 | 1.000 (chi-square test) |
| HBcrAg (log U/mL)[c] | 4.5 (3.6–4.9) | 3.4 (3.0–5.6) | 0.297 |
| HBV DNA (log copies/mL)[c] | 2.2 (2.2–2.2) | 2.2 (2.2–2.7) | 0.562 |
| HBV DNA + RNA titers (log copies/mL) | 3.4 (2.2–4.4) | 2.6 (2.2–3.7) | 0.463 |
| DR ratio | 1.3 (0.2–2.1) | 0.5 (−0.1 to 1.6) | 0.201 |
| Sequential therapy (+:−) | 3:2 | 6:0 | 0.182 (chi-square test) |
| Duration of treatment (weeks)[c] | 31 (24–175) | 24 (24–110) | 0.291 |

[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] Median (range)

ALT rebound rate was lower than that of HBV DNA rebound, but the rate also increased in a time-dependent manner. The cumulative ALT rebound rate reached 41.7 and 71.1 % at 24 and 48 weeks after discontinuation of NA therapy, respectively (Fig. 1b). Accordingly, it was difficult to discontinue NA therapy safely over a long period. Therefore, to identify factors associated with the safe discontinuation of NA therapy, we performed a number of analyses.

## Predictive factors for HBV DNA rebound

To identify those factors associated with HBV DNA rebound, we divided the patients into two groups, namely, a HBV DNA relapse and a non-relapse group, respectively, based on the timing of HBV DNA rebound. The 22 patients whose HBV DNA titers rebounded within 24 weeks after discontinuation of therapy were included in the relapse group, and the remaining 14 patients were included in the non-relapse group. As shown in Table 2, HBV DNA + RNA titers and the DR ratio after 3 months of treatment were both associated with HBV DNA rebound ($P = 0.015$ and $P = 0.019$, respectively). However, duration of treatment and HBsAg, HBcrAg, and HBV DNA levels at the end of treatment were not significant predictive factors. As shown in Fig. 1a, most HBV DNA rebound occurred within 48 weeks of treatment discontinuation. However, subsequent multivariate

**Table 5** Multiple logistic regression for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment in those patients whose HBV DNA did not become negative at the end of NA treatment

| Factors[a] | DNA relapsed ($n = 16$) | DNA non-relapsed ($n = 9$) | Univariate $P$ value[b] | Multiple logistic regression[c] | |
|---|---|---|---|---|---|
| | | | | $P$ value | OR (95 % CI) |
| Gender (M:F) | 9:7 | 3:6 | 0.691 (chi-square test) | | |
| HBV genotype (B:C:ND) | 1:14:1 | 1:7:1 | 0.817 (chi-square test) | | |
| Before treatment | | | | | |
| Age (years)[d] | 41 (25–59) | 39 (30–62) | 0.777 | | |
| Platelet ($\times 10^4/\mu L$)[d] | 17.4 (9.6–28.0) | 14.7 (9.6–18.8) | 0.183 | | |
| ALT (IU/L)[d] | 148 (37–780) | 118 (22–304) | 0.610 | | |
| HBsAg (IU/mL)[d] | 3,730 (462–1,354,400) | 1,384 (66–10,109) | 0.267 | | |
| HBeAg (+:−) | 10:6 | 3:6 | 0.226 (chi-square test) | | |
| HBcrAg (log U/mL)[a] | 6.4 (4.8–8.8) | 6.5 (3.7–7.4) | 0.796 | | |
| HBV DNA (log copies/mL)[d] | 8.4 (3.5–10.1) | 7.7 (4.1–9.2) | 0.294 | | |
| HBV DNA + RNA titers (log copies/mL) | 7.9 (3.8–10.0) | 7.1 (3.8–9.1) | 0.497 | | |
| DR ratio | −0.2 (−1.4 to 0.9) | −0.3 (−1.3 to −0.1) | 0.359 | | |
| After 3 months of treatment | | | | | |
| HBV DNA (log copies/mL)[d] | 4.5 (2.4–7.3) | 3.8 (3.1– 4.6) | 0.118 | | |
| HBV DNA + RNA titers (log copies/mL) | 5.6 (3.7–8.2) | 4.7 (2.4–6.2) | 0.089 | 0.068 | 2.048 (0.949–4.419) |
| DR ratio | 1.0 (−0.6 to 2.7) | 0.0 (−0.7 to 1.4) | 0.061 | 0.320 | |
| End of treatment | | | | | |
| HBsAg (IU/mL)[d] | 2,306 (481–11,607) | 626 (<1.1–9,680) | 0.064 | 0.839 | |
| HBeAg (+:−) | 10:6 | 2:7 | 0.097 (chi-square test) | 0.490 | |
| HBcrAg (log U/mL)[d] | 5.1 (3.0–8.2) | 5.1 (3.1–6.6) | 1.000 | | |
| HBV DNA (log copies/mL)[d] | 3.9 (2.8–9.2) | 4.1 (2.8–7.1) | 0.887 | | |
| HBV DNA + RNA titers (log copies/mL) | 4.2 (3.1– 8.7) | 3.9 (2.2–6.5) | 0.411 | | |
| DR ratio | 0.3 (−1.0 to 2.8) | −0.4 (−0.8 to 1.2) | 0.061 | 0.171 | |
| Sequential therapy (+:−) | 10:6 | 7:2 | 0.661 (chi-square test) | | |
| Duration of treatment (weeks)[d] | 35 (24–221) | 86 (24–304) | 0.164 | | |

[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)

analysis aimed at identifying factors associated with HBV DNA rebound within 48 weeks after discontinuation of therapy did not identify any independent factors (Table 3).

Because HBV DNA rebound is assumed to be associated with HBV replication activity, HBV DNA and HBV DNA + RNA titers were compared at several points during treatment (Fig. 2). In the non-relapse group, HBV DNA and HBV DNA + RNA titers decreased rapidly, and no divergence was observed during NA therapy (Fig. 2a). In comparison, while HBV DNA titer also declined rapidly in the relapse group, the reduction in HBV DNA + RNA titers occurred so gradually that the two titers had significantly diverged by 2 months after the start of treatment (Fig. 2b).

Multivariate analysis of HBV DNA rebound was performed using the following candidate factors: HBsAg and HBeAg before nucleotide treatment, HBV DNA, HBV

**Table 6** Multiple logistic regression for factors associated with ALT rebound within 24 weeks after discontinuation of NA treatment

| Factors[a] | ALT relapsed ($n = 13$) | ALT non-relapsed ($n = 23$) | Univariate $P$ value[b] | Multiple logistic regression[c] | |
|---|---|---|---|---|---|
| | | | | $P$ value | OR (95 % CI) |
| Gender (M:F) | 7:6 | 16:7 | 0.346 (chi-square test) | | |
| HBV genotype (B:C:ND) | 0:12:1 | 2:19:2 | 0.540 (chi-square test) | | |
| Before treatment | | | | | |
| Age (years)[d] | 40 (25–59) | 47 (29–66) | 0.149 | | |
| Platelet ($\times 10^4/\mu L$)[d] | 19.1 (9.6–28.0) | 14.8 (9.6–27.5) | 0.205 | | |
| ALT (IU/L)[d] | 35 (37–309) | 143 (22–780) | 0.795 | | |
| HBsAg (IU/mL)[d] | 3,730 (462–1,354,400) | 2,092 (66–10,109) | 0.127 | | |
| HBeAg (+:−) | 10:3 | 6:17 | 0.005 (chi-square test) | 0.544 | |
| HBcrAg (log U/mL)[d] | 6.4 (5.5–8.8) | 5.4 (3.4–7.9) | 0.131 | | |
| HBV DNA (log copies/mL)[d] | 7.7 (5.0–10.1) | 7.7 (3.5–9.7) | 0.434 | | |
| HBV DNA + RNA titers (log copies/mL) | 7.8 (5.1–10.0) | 7.5 (3.4–9.7) | 0.397 | | |
| DR ratio | −0.2 (−1.4 to 0.9) | −0.4 (−1.4 to 0.5) | 0.336 | | |
| After 3 months of treatment | | | | | |
| HBV DNA (log copies/mL)[d] | 4.9 (2.4–7.3) | 3.7 (2.2–4.8) | 0.007 | 0.228 | |
| HBV DNA + RNA titers (log copies/mL) | 5.7 (3.8–8.2) | 4.1 (2.2–6.3) | 0.004 | 0.120 | |
| DR ratio | 0.9 (−0.2 to 2.7) | 0.6 (−0.9 to 1.9) | 0.115 | | |
| End of treatment | | | | | |
| HBsAg (IU/mL)[d] | 2,306 (481–11,607) | 824 (<1.1–11,600) | 0.019 | 0.821 | |
| HBeAg (+:−) | 10:3 | 4:19 | 0.001 (chi-square test) | 0.003 | 13.500 (2.473–73.705) |
| HBcrAg (log U/mL)[d] | 5.4 (3.6–8.2) | 4.3 (3.0–6.6) | 0.085 | 0.264 | |
| HBV DNA (log copies/mL)[d] | 4.4 (2.2–9.2) | 3.3 (2.2–7.1) | 0.070 | 0.380 | |
| HBV DNA + RNA titers (log copies/mL) | 4.4 (3.1–8.7) | 3.6 (2.2–6.5) | 0.004 | 0.174 | |
| DR ratio | 0.4 (−1.0 to 2.8) | 0.2 (−0.8 to 1.6) | 0.434 | | |
| Sequential therapy (+:−) | 9:4 | 17:6 | 0.527 (chi-square test) | | |
| Duration of treatment (weeks)[d] | 29 (24–221) | 51 (24–304) | 0.169 | | |

[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)

DNA + RNA titers, and DR ratio after 3 months of treatment, and HBsAg and HBeAg at the end of treatment. As shown in Table 2, only HBV DNA + RNA titer after 3 months of treatment was identified as an independent predictive factor for the safe discontinuation of NA therapy without HBV DNA rebound ($P = 0.043$, OR 9.474, 95 % CI 1.069–83.957). HBsAg titer at the end of treatment and HBV DNA titer after 3 months of treatment were marginally associated ($P = 0.070$, $P = 0.074$, respectively). These results suggest that HBV rebound is significantly associated with HBV replication activity during NA treatment.

To analyze the cumulative HBV DNA rebound rate, we divided the 36 subjects into two groups. Cut-off values for assigning patients to the groups were determined by inspection of the receiver operating characteristic (ROC) curve. According to this curve, the best cut-off value of HBV DNA + RNA after 3 months of treatment was

**Table 7** Multiple logistic regression for factors associated with ALT rebound within 48 weeks after discontinuation of NA treatment

| Factors[a] | ALT relapsed ($n = 25$) | ALT non-relapsed ($n = 11$) | Univariate $P$ value[b] | Multiple logistic regression[c] | |
|---|---|---|---|---|---|
| | | | | $P$ value | OR (95 % CI) |
| Gender (M:F) | 17:8 | 6:5 | 0.475 (chi-square test) | | |
| HBV genotype (B:C:ND) | 2:21:2 | 0:10:1 | 0.627 (chi-square test) | | |
| Before treatment | | | | | |
| Age (years)[d] | 41 (25–64) | 45 (29–66) | 0.877 | | |
| Platelet ($\times 10^4/\mu L$)[d] | 15.6 (9.6–28.0) | 16.5 (9.6–27.5) | 0.768 | | |
| ALT (IU/L)[d] | 143 (22–402) | 118 (48–780) | 0.945 | | |
| HBsAg (IU/mL)[d] | 2,878 (66–1,354,400) | 4,908 (1,172–10,109) | 0.490 | | |
| HBeAg (+:−) | 12:13 | 4:7 | 0.718 (chi-square test) | | |
| HBcrAg (log U/mL)[d] | 6.3 (4.0–8.8) | 5.8 (3.4–7.9) | 0.518 | | |
| HBV DNA (log copies/mL)[d] | 7.7 (3.5–10.1) | 7.7 (3.8–9.6) | 0.353 | | |
| HBV DNA + RNA titers (log copies/mL) | 7.8 (3.8–10.0) | 7.4 (3.4–9.0) | 0.429 | | |
| DR ratio | −0.2 (−1.4 to 0.9) | −0.4 (−1.3 to 0.5) | 0.201 | | |
| After 3 months of treatment | | | | | |
| HBV DNA (log copies/mL)[d] | 4.2 (2.2–7.3) | 3.6 (2.2–4.6) | 0.082 | 0.106 | |
| HBV DNA + RNA titers (log copies/mL) | 4.8 (2.2–8.2) | 4.2 (2.2–6.3) | 0.271 | | |
| DR ratio | 0.7 (−0.9 to 2.7) | 0.6 (−0.7 to 1.9) | 0.757 | | |
| End of treatment | | | | | |
| HBsAg (IU/mL)[d] | 2,387 (48–16,301) | 812 (<1.1–11,600) | 0.183 | | |
| HBeAg (+:−) | 13:12 | 2:9 | 0.142 (chi-square test) | | |
| HBcrAg (log U/mL)[d] | 5.1 (3.0–8.2) | 3.9 (3.0–6.6) | 0.291 | | |
| HBV DNA (log copies/mL)[d] | 3.6 (2.1–9.2) | 3.3 (2.2–7.1) | 0.782 | | |
| HBV DNA + RNA titers (log copies/mL) | 3.7 (2.2–8.7) | 3.6 (2.2–6.5) | 0.655 | | |
| DR ratio | 0.3 (−1.0 to 2.8) | −0.1 (−0.8 to 1.3) | 0.135 | | |
| Sequential therapy (+:−) | 20:5 | 6:5 | 0.224 (chi-square test) | | |
| Duration of treatment (weeks)[d] | 31 (24–221) | 91 (24–304) | 0.028 | 0.034 | 1.014 (1.001–1.027) |

[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)

4.8 log copies/mL (sensitivity 0.733, specificity 0.619, positive predictive value 0.578, negative predictive value 0.765). Seventeen subjects who achieved a titer of <4.8 log copies/mL of HBV DNA + RNA after 3 months of treatment were assigned to group A; the remaining 19 subjects were assigned to group B. The cumulative HBV DNA rebound rate of group A was significantly lower than that of group B at 24 weeks after discontinuation ($P = 0.045$, Fig. 3).

To address potential bias in the study criteria, we analyzed subjects separately depending on whether HBV DNA titer became negative or not at the end of treatment to identify factors associated with HBV DNA rebound. No significant factors for HBV DNA rebound were identified in patients whose HBV DNA titer became negative at the end of NA treatment ($n = 11$) (Table 4). In patients whose HBV DNA did not become negative at the end of NA treatment ($n = 25$), HBV DNA + RNA titer after

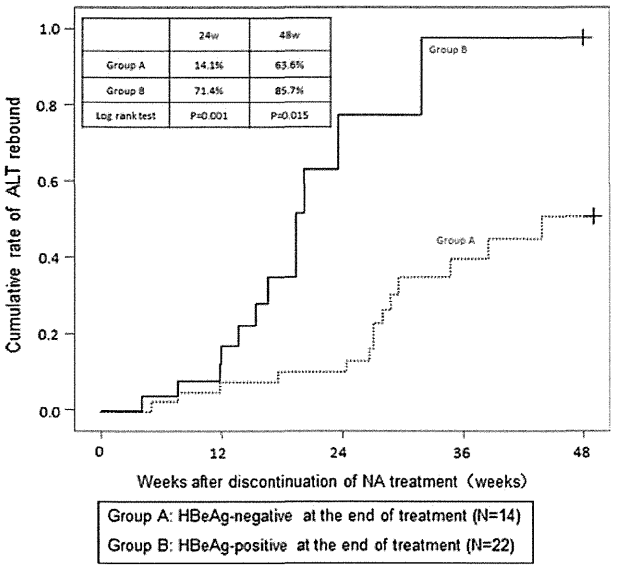


**Fig. 4** Cumulative rate of ALT rebound after discontinuation of NA treatment. Fourteen patients who were hepatitis B virus e antigen (*HBeAg*) negative at the end of treatment were assigned to group A; the other 22 patients, who were positive to HBeAg at the end of treatment, were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

3 months of treatment was identified as a marginally significant predictive factor for safe discontinuation of NA therapy without HBV DNA rebound ($P = 0.068$, OR 2.048, 95 % CI 0.949–4.419) (Table 5).

## Predictive factors for ALT rebound

To identify predictive factors for ALT rebound, patients were divided into two groups based on the timing of ALT elevation. The 13 patients whose ALT levels rebounded within 24 weeks after discontinuation of therapy were assigned to the ALT relapse group, and the remaining 23 patients were assigned to the ALT non-relapse group. As shown in Table 6, HBeAg presence before treatment, HBV DNA and HBV DNA + RNA titers after 3 months of treatment, and HBeAg presence, HBV DNA + RNA levels, and HBsAg titer at the end of treatment were significantly associated with ALT relapse in the univariate analysis. However, ALT, duration of treatment, and DR ratio at the end of treatment were not significant.

As shown in Table 6, multivariate analysis of ALT rebound was performed using the following candidate factors: HBeAg presence before treatment, HBV DNA and HBV DNA + RNA levels after 3 months of treatment, and HBeAg presence, HBV DNA and DNA + RNA levels, HBcrAg titer, and HBsAg titer at the end of treatment. Only the presence of HBeAg at the end of treatment was identified as an independent predictive factor for safe discontinuation of NA therapy without ALT rebound ($P = 0.003$, OR 13.500, 95 % CI 2.473–73.705). These results suggest that ALT rebound is also significantly associated with HBV replication activity during NA therapy.

As shown in Fig. 1b, most ALT rebound also occurred within 48 weeks. We performed further analysis to identify factors associated with ALT rebound within 48 weeks after discontinuation of NA therapy. In the univariate analysis, duration of NA treatment was significantly associated with ALT relapse, and HBV DNA level after 3 months of treatment was marginally associated with ALT relapse. Only duration of NA treatment was identified as an independent predictive factor for safe discontinuation of NA therapy without ALT rebound by multivariate analysis ($P = 0.034$, OR 1.014, 95 % CI 1.001–1.027) (Table 7).

To analyze the cumulative ALT rebound rate, the 36 subjects were divided into two groups based on HBeAg presence. Twenty-two subjects who were HBeAg-negative at the end of treatment were assigned to group A, and the remaining 14 subjects were assigned to group B. The cumulative ALT rebound rate of group A was significantly lower than that of group B at 24 and 48 weeks after discontinuation of therapy ($P = 0.001$, $P = 0.015$, respectively; Fig. 4).

To account for potential bias in the study criteria, we analyzed subjects separately based on whether ALT was normalized or not at the end of treatment, with the aim of identifying factors for ALT rebound. In patients whose ALT was normalized at the end of NA treatment ($n = 25$), HBeAg presence before treatment, HBV DNA and HBV DNA + RNA titers after 3 months of treatment, and HBeAg presence at the end of treatment were significantly associated with ALT relapse in the univariate analysis. HBeAg presence at the end of treatment was identified as an independent predictive factor for safe discontinuation of NA therapy without ALT relapse (Table 8). In patients whose ALT was not normalized at the end of NA treatment ($n = 11$), only HBV DNA titer after 3 months of treatment was marginally associated with ALT relapse in the univariate analysis ($P = 0.052$; Table 9).

## Predictive factors for ALT rebound in HBeAg-positive patients

Because the cumulative rate of ALT rebound in HBeAg-positive CHB patients was significantly higher thanthat in HBeAg-negative patients, we focused on the 16 HBeAg-positive patients to identify factors associated with ALT rebound in these patients. As shown in Table 10, only the HBV DNA + RNA titer after 3 months of treatment was significant in the univariate analysis. However, in multivariate analysis, the HBV DNA + RNA titer after

**Table 8** Multiple logistic regression for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment in those patients whose ALT levels had normalized at the end of NA treatment

| Factors[a] | ALT relapsed ($n = 6$) | ALT non-relapsed ($n = 19$) | Univariate $P$ value[b] | Multiple logistic regression[c] | |
|---|---|---|---|---|---|
| | | | | $P$ value | OR (95 % CI) |
| Gender (M:F) | 5:1 | 12:7 | 0.073 (chi-square test) | 0.073 | |
| HBV genotype (B:C:ND) | 0:6:0 | 2:16:1 | 0.584 (chi-square test) | | |
| Before treatment | | | | | |
| Age (years)[d] | 41 (31–59) | 46 (29–66) | 0.545 | | |
| Platelet ($\times 10^4/\mu L$)[d] | 20.3 (9.6–28.0) | 14.7 (9.6–27.5) | 0.484 | | |
| ALT (IU/L)[d] | 161 (62–309) | 118 (22–780) | 0.750 | | |
| HBsAg (IU/mL)[d] | 3,573 (462–1,354,400) | 2,485 (66–0.109) | 0.201 | | |
| HBeAg (+:−) | 5:1 | 5:14 | 0.023 (chi-square test) | 0.707 | |
| HBcrAg (log U/mL)[d] | 7.1 (6.5–7.8) | 5.3 (3.4–7.9) | 0.264 | | |
| HBV DNA (log copies/mL)[d] | 9.1 (6.8–10.0) | 8.1 (3.5–9.6) | 0.252 | | |
| HBV DNA + RNA titers (log copies/mL) | 8.3 (6.1–9.7) | 7.5 (3.4–9.2) | 0.477 | | |
| DR ratio | −0.5 (−1.4 to 0.0) | −0.4 (−1.4 to 0.5) | 0.503 | | |
| After 3 months of treatment | | | | | |
| HBV DNA (log copies/mL)[d] | 3.7 (2.4–6.9) | 3.7 (2.2–4.8) | 0.503 | | |
| HBV DNA + RNA titers (log copies/mL) | 3.7 (2.4–6.9) | 4.2 (2.2–6.3) | 0.041 | 0.413 | |
| DR ratio | 1.4 (−0.2 to 1.9) | 0.7 (−0.9 to 1.9) | 0.111 | | |
| End of treatment | | | | | |
| HBsAg (IU/mL)[d] | 2,978 (481–16,301) | 812 (<1.1–11,600) | 0.127 | | |
| HBeAg (+:−) | 5:1 | 3:16 | 0.006 (chi-square test) | 0.009 | 26.667 (2.242–317.147) |
| HBcrAg (log U/mL)[d] | 4.1 (3.6–5.8) | 3.7 (3.0–6.6) | 0.406 | | |
| HBV DNA (log copies/mL)[d] | 3.3 (2.2–6.3) | 3.4 (2.2–6.1) | 0.632 | | |
| HBV DNA + RNA titers (log copies/mL) | 4.1 (3.2–7.1) | 3.6 (2.2–5.7) | 0.064 | 0.444 | |
| DR ratio | 0.6 (−1.0 to 2.8) | 0.2 (−0.8 to 1.5) | 0.340 | | |
| Sequential therapy (+:−) | 3:3 | 13:6 | 0.630 (chi-square test) | | |
| Duration of treatment (weeks)[d] | 59 (25–221) | 51 (24–304) | 0.702 | | |

[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)

3 months of treatment was only marginally associated with the safe discontinuation of NA therapy without ALT rebound ($P = 0.050$, OR 8.032, 95 % CI 0.997–64.683). These results suggest that ALT rebound in HBeAg-positive patients might be associated with HBV replication activity during the NA treatment.

To analyze the cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients, the 16 subjects were divided into two groups based on HBV DNA + RNA levels. The cut-off value of HBV DNA + RNA after 3 months of treatment (4.8 log copies/mL) was determined by inspection of the ROC curve (sensitivity 0.833, specificity: 0.889, positive predictive value 0.833, negative predictive value 0.889). Six subjects who achieved <5.0 log copies/mL of HBV DNA + RNA levels after 3 months of treatment were assigned to group A and the remaining

**Table 9** Univariate analysis for factors associated with HBV DNA rebound within 24 weeks after discontinuation of NA treatment in the patients in whom ALT levels did not normalize at the end of NA treatment

| Factors | ALT relapsed ($n = 7$) | ALT non-relapsed ($n = 4$) | Univariate $P$ value |
|---|---|---|---|
| Gender (M:F) | 6:1 | 4:0 | 1.000[b] |
| HBV genotype (B:C:ND) | 0:6:1 | 0:3:1 | 1.000[b] |
| Before treatment | | | |
| Age (years)[a] | 36 (25–56) | 50 (30–64) | 0.218 |
| Platelet ($\times 10^4/\mu L$)[a] | 17.0 (13.1–27.5) | 16.1 (15.6–16.5) | 0.770 |
| ALT (IU/L)[a] | 101 (37–303) | 148 (114–270) | 0.571 |
| HBsAg (IU/mL)[a] | 11,113 (1,180–40,967) | 1,384 (406–7,016) | 0.197 |
| HBeAg (+: −) | 5:2 | 1:3 | 0.242[b] |
| HBcrAg (log U/mL)[a] | 5.9 (5.5–8.8) | 6.7 (5.0–7.7) | 1.000 |
| HBV DNA (log copies/mL)[a] | 7.1 (5.0–10.1) | 6.7 (5.7–9.7) | 0.635 |
| HBV DNA + RNA titers (log copies/mL) | 6.9 (5.1–10.0) | 6.3 (5.0–9.7) | 0.571 |
| DR ratio | −0.1 (−0.2–0.9) | −0.4 (−0.7–0.0) | 0.279 |
| After 3 months of treatment | | | |
| HBV DNA (log copies/mL)[a] | 5.1 (3.8–7.3) | 4.2 (2.2–4.4) | 0.052 |
| HBV DNA + RNA titers (log copies/mL) | 5.7 (3.9–8.2) | 4.4 (2.9–6.2) | 0.185 |
| DR ratio | 0.6 (−0.2–2.7) | 0.1 (−0.1–0.6) | 0.255 |
| End of treatment | | | |
| HBsAg (IU/mL)[a] | 4,317 (2,306–11,607) | 5,209 (85–5,711) | 0.915 |
| HBeAg (+: −) | 5:2 | 1:3 | 0.242[b] |
| HBcrAg (log U/mL)[a] | 5.4 (3.6–8.2) | 5.6 (4.9–5.9) | 1.000 |
| HBV DNA (log copies/mL)[a] | 4.4 (2.2–9.2) | 2.2 (2.2–7.1) | 0.178 |
| HBV DNA + RNA titers (log copies/mL) | 4.9 (3.1–8.7) | 3.0 (2.2–6.5) | 0.131 |
| DR ratio | −0.1 (−0.5–2.7) | 0.1 (−0.6–1.6) | 0.850 |
| Sequential therapy (+: −) | 6;1 | 4:0 | 1.000[b] |
| Duration of treatment (weeks)[a] | 24 (24–36) | 44 (24–110) | 0.091 |

*ND* not determined, *DR ratio* HBV DNA + RNA titers/HBV DNA

[a] Median (range) univariate analysis was performed with Mann-Whitney *U* test

[b] Chi-square test

ten subjects were assigned to group B. The cumulative ALT rebound rate of group A was significantly lower than that of group B at 24 and 48 weeks after the discontinuation of therapy ($P = 0.008$, $P = 0.024$, respectively, Fig. 5).

### Prediction of ALT rebound after discontinuation of therapy using two extracted factors

To predict successful discontinuation of therapy, we analyzed cumulative ALT rebound by using HBV DNA plus RNA levels at 3 months of NA treatment and existence of HBeAg at the end of treatment. Fourteen subjects who achieved both <4.8 log copies/mL of HBV DNA + RNA levels after 3 months of treatment and negative HBeAg at the end of treatment were assigned to group A and the remaining 22 subjects were assigned to group B. The cumulative ALT rebound rate of group A was significantly lower than that of group B among all observation periods ($P = 0.046$, Fig. 6).

## Discussion

Since the introduction of NAs, chronic hepatitis B progression has been drastically suppressed. NAs strongly suppress HBV replication in human hepatocytes and rapidly decrease serum HBV DNA titers to undetectable levels [30–33]. However, even if HBV DNA is continuously maintained at undetectable levels, it is difficult to

**Table 10** Multiple logistic regression for factors associated with ALT rebound within 24 weeks after discontinuation of NA therapy in HBeAg-positive patients ($n = 16$)

| Factors[a] | ALT relapsed ($N = 10$) | ALT non-relapsed ($N = 6$) | Univariate $P$ value[b] | Multiple logistic regression[c] | |
|---|---|---|---|---|---|
| | | | | $P$ value | OR (95 % CI) |
| Gender (M:F) | 5:5 | 3:3 | 0.696 (chi-square test) | | |
| HBV genotype (B:C) | 0:10 | 0:6 | 1.000 (chi-square test) | | |
| Before treatment | | | | | |
| Age (years)[d] | 35 (25–56) | 38 (29–47) | 0.957 | | |
| Platelets ($\times 10^4/\mu L$)[d] | 20.3 (9.6–28.0) | 17.3 (14.5–27.5) | 0.768 | | |
| ALT (IU/L)[d] | 148 (37–309) | 155 (46–270) | 0.958 | | |
| HBsAg (IU/mL)[d] | 11,113 (462–1,354,400) | 6,283 (66–10,109) | 0.662 | | |
| HBcrAg (log U/mL)[d] | 7.1 (5.5–8.8) | 7.4 (5.2–7.7) | 0.714 | | |
| HBV DNA (log copies/mL)[d] | 9.1 (6.5–10.1) | 8.8 (3.8–9.7) | 0.792 | | |
| HBV DNA + RNA titers (log copies/mL) | 8.3 (6.1–10.0) | 8.6 (3.4–9.7) | 0.958 | | |
| DR ratio | −0.2 (−1.4 to 0.9) | −0.3 (−0.7 to 0.0) | 0.776 | | |
| After 3 months of treatment | | | | | |
| HBV DNA (log copies/mL)[d] | 5.0 (3.5–7.3) | 4.1 (2.2–4.4) | 0.056 | 0.897 | |
| HBV DNA + RNA titers (log copies/mL) | 5.8 (4.8–8.2) | 4.7 (3.7–6.3) | 0.011 | 0.050 | 8.032 (0.997–64.683) |
| DR ratio | 1.1 (−0.2 to 2.7) | 1.1 (−0.6 to 1.9) | 0.792 | | |
| End of treatment | | | | | |
| HBsAg (IU/mL)[d] | 4,736 (823–16,301) | 3,523 (48–11,600) | 0.529 | | |
| HBeAg (+:−) | 10:0 | 4:2 | 0.125 (chi-square test) | | |
| HBcrAg (log U/mL)[d] | 5.6 (4.1–8.2) | 5.3 (4.0–6.6) | 0.310 | | |
| HBV DNA (log copies/mL)[d] | 4.4 (2.2–9.2) | 3.7 (2.1–6.1) | 0.220 | | |
| HBV DNA + RNA titers (log copies/mL) | 4.9 (3.7–8.7) | 3.9 (3.4–5.7) | 0.093 | 0.543 | |
| DR ratio | 0.5 (−1.0 to 2.8) | 0.2 (−0.8 to 1.6) | 0.635 | | |
| Sequential therapy (+:−) | 7:3 | 4:2 | 0.654 (chi-square test) | | |
| Duration of treatment (weeks)[d] | 29 (24– 221) | 119 (24–175) | 0.169 | | |

[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)

completely eliminate HBV from the liver. The goal of NA therapy is therefore to reduce the HBV DNA titer and to induce an inactive state of hepatitis, but, as a result, it is necessary that NA therapy should be continued for a long period of time. As it is well known that long-term treatment with NAs increases the incidence of HBV drug resistance [14], we propose that patients who maintain an inactive state of hepatitis with NA therapy may be able to discontinue the NA therapy to prevent the appearance of drug-resistant strains. However, as shown in Fig. 1, in our patient cohort, hepatitis was re-activated after discontinuation of the therapy in more than 70 % of the patients who discontinued the NA therapy. Therefore, in this study, we analyzed predictive factors for the safe discontinuation of NA therapy.

After discontinuation of NA therapy, serum HBV DNA titers increased in 91.7 % of our patients within 48 weeks (Fig. 1a). In the multivariate logistic regression, the HBV