Guidelines proposed by the Japanese Study Group of the Standardization of Treatment of Viral Hepatitis [49] basically recommend IFN as the first-line treatment for chronic hepatitis B patients aged <35 years to attain a "drug-free state" and entecavir for patients aged ≥35 years to persistently suppress HBV DNA (as tenofovir disoproxil fumarate has not been licensed in Japan to date). In patients aged <35 years and harboring HBV DNA in titers of ≥7 log copies/mL, sequential treatment with entecavir followed by IFN is recommended as the first-line therapy if HBeAg is negative and as the second-line therapy (next to IFN monotherapy) if HBeAg is positive. In patients aged ≥35 years and harboring HBV DNA of ≥7 log copies/mL, sequential treatment is recommended as the second-line therapy (next to entecavir) if the HBeAg is positive.

Conclusions

It remains unclear whether combination therapy is superior to monotherapy for treating chronic hepatitis B. Consequently, controlled trials comparing combination and monotherapy are necessary. In previous studies comparing the lamivudine + IFN combination and lamivudine monotherapy in a finite course, combination therapy was associated with higher rates of sustained post-treatment response and lower rates of drug resistance than lamivudine monotherapy. However, NAs are generally administered indefinitely due to high rates of post-treatment relapse. Additionally, even when NAs are administered alone, concern for drug resistance has significantly decreased (≤1.2 % in 3–5 years [50, 51]) with the development of newer high-potency NAs, such as entecavir and tenofovir. In previous studies comparing the lamivudine + IFN combination and IFN monotherapy, combination therapy showed greater on-treatment viral suppression, but no difference in the post-treatment sustained response was observed when compared to therapy with IFN alone. The efficacy of combining IFN with a more potent NA remains to be evaluated. Further studies are needed to determine whether switching to IFN contributes to the safe discontinuation of therapy, particularly in patients with decreased HBsAg and/or HBcrAg levels during long-term NA treatment [52].

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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.

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

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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 × 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 × 10^6 copies/mL) or genotype 1b HCV (2.2 × 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 (⩾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

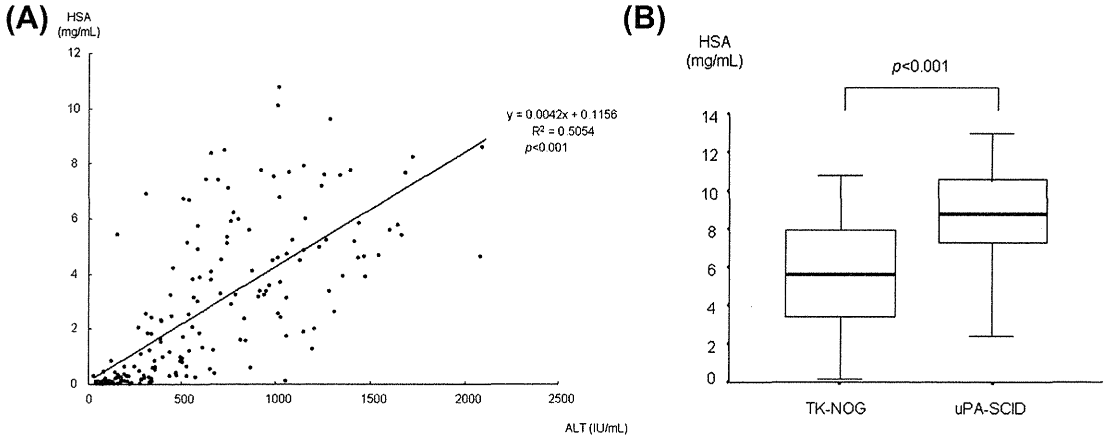

Fig. 1. Human hepatocyte repopulation index in humanized mice. Serum alaninaminotransferase (ALT) levels in TK-NOG mice were measured one week after ganciclovir treatment. Human serum albumin (HSA) levels were measured eight weeks after transplantation of human hepatocytes. (A) Correlation between serum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

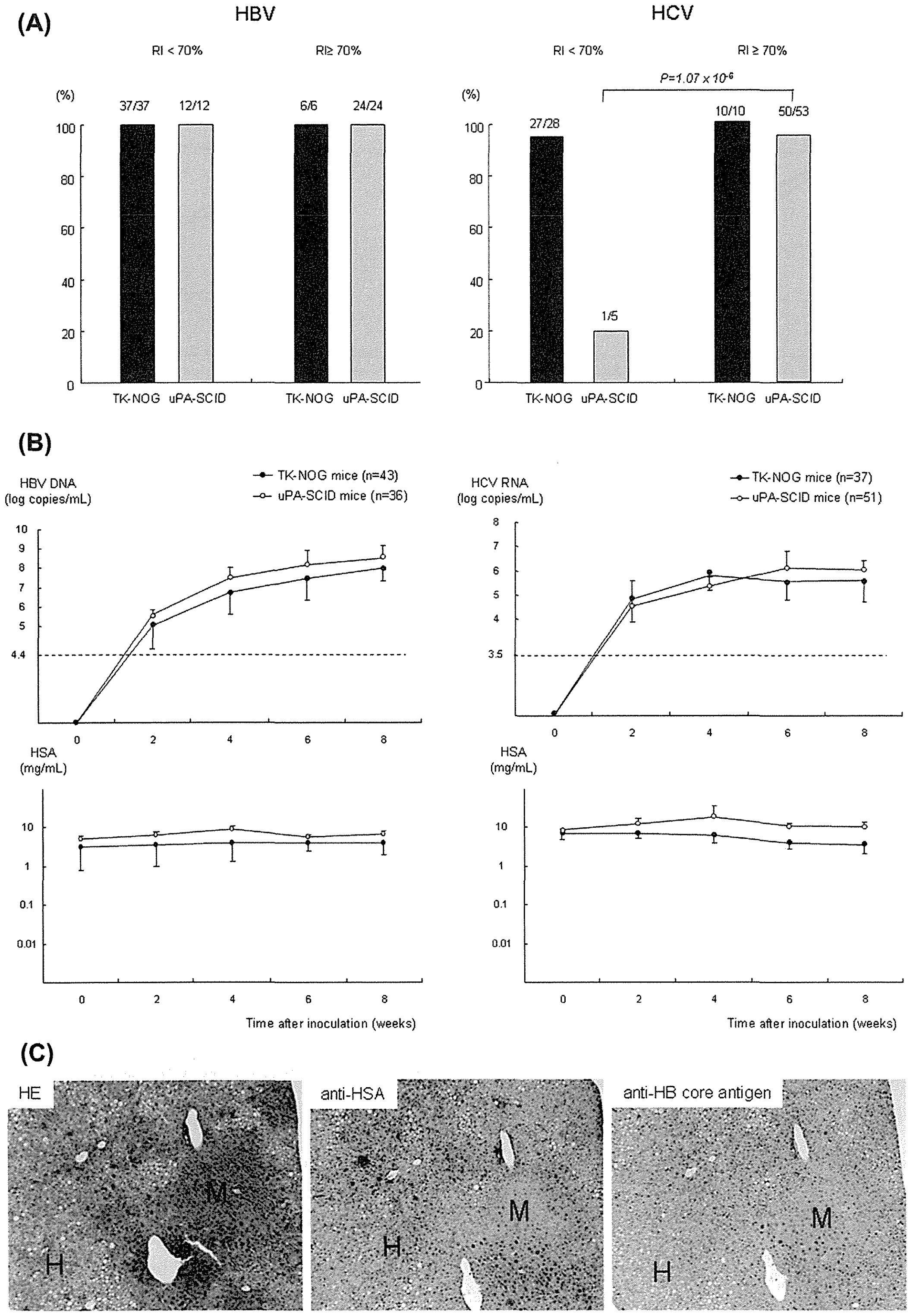

Fig. 2. Hepatitis viruses infection in chimeric mice. (A) Eight weeks after human hepatocyte transplantation, mice with serum HSA level over 1 mg/mL were inoculated with HBV- or HCV-positive human serum samples. Percentages of mice that became positive for HBV DNA (left panel) or HCV RNA (right panel)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

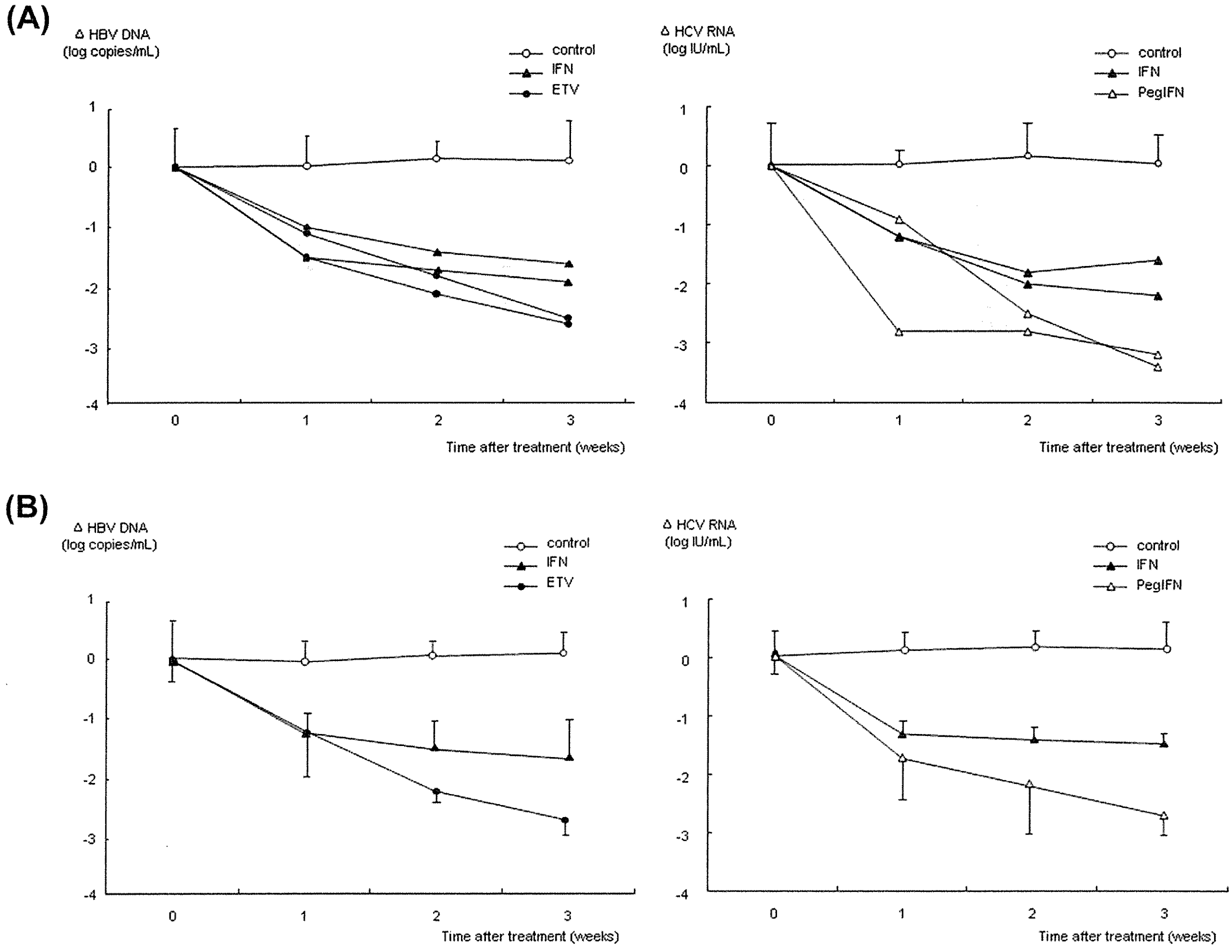

Fig. 3. Reduction of serum viral titers in mice treated with anti-viral agents. (A) HBV- (left panel) or HCV-infected (right panel) TK-NOG mice were treated with entecavir, interferon (IFN)-alpha or PegIFN-alpha-2a. Control: HBV- and HCV-infected mice without antiviral treatment. (B) HBV- (left panel)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

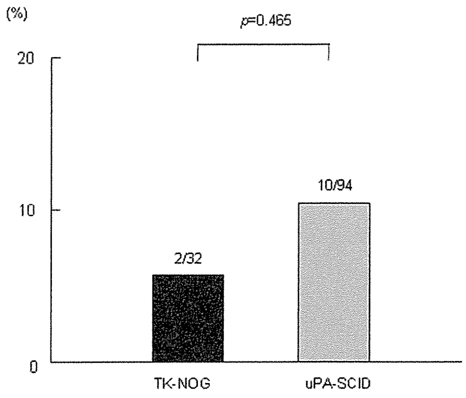

Fig. 4. Frequency of unexpected death within 8 weeks in mice. The numbers of sudden deaths occurring within 8 weeks of viral infection in TK-NOG mice and uPA-SCID mice are shown as bars.

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.

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LETTER TO THE EDITOR

Availability of monitoring serum HBV DNA plus RNA during nucleot(s)ide analogue therapy

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We appreciate the comments by Kurosaki et al. on the article entitled "Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleot(s)ide analogue (NUC) treatments in chronic hepatitis B patients" [1]. They raised three important questions: (1) whether HBV DNA levels measured by transcription-mediated amplification and hybridization (TMA-HPA) can be used as an alternative to HBV DNA plus RNA levels measured by RT-PCR; (2) whether post-treatment monitoring of serum HBV DNA plus RNA might serve as a predictor of safe discontinuation after long term NUC; and (3) whether serum HBV DNA plus RNA titer is a predictor of favorable response to sequential interferon therapy.

The presence of HBV RNA in serum is an indicator of ongoing transcription of the HBV pregenome from cccDNA in hepatocytes and may occur even when production of mature HBV particles is effectively suppressed by inhibition of reverse transcription by NUC. As we previously reported, lamivudine resistant strains emerge more easily under such conditions [2], but HBV RNA gradually decreases under continued suppression of reverse transcription and generally becomes undetectable in patients following a year of NUC treatment.

The first question Kurosaki et al. was whether HBV DNA titers measured by TMA-HPA assay, which actually represent HBV DNA plus RNA titers, can be used as an alternative to HBV DNA plus RNA measured by RT-PCR. As we showed in our previous report [2], levels obtained by TMA-PHA assay correlated well with those obtained by RT-PCR during NUC therapy ($r = 0.955$, $P < 0.0001$) [2]. Therefore, measurement of TMA-PHA is a reasonable alternative to RT-PCR. Although the sensitivity of HBV nucleic acids by TMA-HPA assay is lower than RT-PCR, measurement of HBV nucleic acids may provide useful information, especially for those patients who started NUC therapy with high pretreatment HBV DNA levels. RT-PCR is more useful in patients who had relatively lower HBV levels at the beginning of NUC therapy.

The second question was whether monitoring of serum HBV DNA plus RNA at the end of treatment serves as a predictor of safe discontinuation after long term NUC. We found that HBV RNA can be detected in patients who became negative for HBV DNA after long term NUC therapy, and measurement of HBV RNA in patients receiving long term NUC therapy may yield important insight into the risk of reactivation of HBV if NUC therapy is discontinued. However, we have not analyzed enough such patients, and a prospective study is necessary to evaluate the predictive value of HBV RNA plus RNA measurement.

The third question was whether serum HBV DNA plus RNA titer is a predictor of favorable response to sequential NUC and interferon therapy. The mechanisms of these drugs is different, and interferon is not associated with serum HBV RNA because it does not disturb reverse

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transcription but instead suppresses HBV transcription in hepatocytes. In our previous study [3], HBV RNA was negative before administration of NUC and became positive soon after the beginning of NUC therapy, peaking at weeks two to four and then gradually decreasing. We assumed that, after HBV RNA levels have been reduced during long term NUC therapy, HBV RNA should become undetectable during interferon therapy [3]. We tried to assess the predictive effect of HBV RNA titer immediately prior to interferon administration in patients who received sequential therapy, but, incidentally, HBV RNA was undetectable in all patients just before interferon treatment [3]. As we did not show results for sequential therapy in our study in Journal of Gastroenterology [1], results of the 26 patients (20 males, 6 females) who underwent sequential therapy patients in that study are described below. Ten patients were positive for HBeAg at the end of NUC therapy. HBV DNA rebound was observed in 13 patients within 24 weeks after discontinuation of NUC therapy, and ALT rebound occurred in 9 patients. HBV DNA rebound was significantly associated with serum HBV DNA plus RNA titer following 3 months of NUC treatment ($P = 0.029$, Mann–Whitney U test), and ALT rebound was significantly associated with serum HBV DNA titer and DNA plus RNA titer following 3 months of NUC treatment ($P = 0.041$, $P = 0.016$, respectively, Mann–Whitney U test) and the existence of HBeAg at the end of NUC treatment ($P = 0.009$, Fisher's exact test). Although it is necessary to confirm these results in a large, prospective study, we conclude from these results that HBV RNA plus DNA is a predictor for sequential therapy.

Due to the complicated nature of chronic HBV infection and immunological reaction of the host, it is difficult to completely predict the effect of any type of therapy. Further study should be done to identify conditions for safe discontinuation of NUC because otherwise patients must continue lifelong NUC therapy. We thank Kurosaki et al. for their helpful comments and appreciate the opportunity to respond to their questions.

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Hepatology Research

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Original Article

Utility of controlled attenuation parameter measurement for assessing liver steatosis in Japanese patients with chronic liver diseases

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Aim: Steatosis is a common histological feature of chronic liver disease, especially alcoholic and non-alcoholic fatty liver disease, as well as chronic hepatitis C. A recent study showed that evaluating the controlled attenuation parameter (CAP) with transient elastography was an efficient way of non-invasively determining the severity of hepatic steatosis. The objective of this study was to prospectively evaluate the utility of CAP for diagnosing steatosis in patients with chronic liver disease.

Methods: One hundred and fifty-five consecutive patients with suspected chronic liver disease underwent steatosis diagnosis using CAP, blood sample analyses, computed tomography for assessing the liver/spleen ratio and liver biopsy. Steatosis was graded according to the percentage of fat-containing hepatocytes: S0, less than 5%; S1, 5–33%; S2, 34–66%; and S3: more than 66%.

Results: The CAP was significantly correlated with steatosis grade, and there were significant differences between the CAP value of the S0 patients and those of the patients with other grades of steatosis. S0 and S1–3 hepatic steatosis were considered to represent mild and significant steatosis, respectively. The CAP values of the patients with mild and significant steatosis were significantly different ($P < 0.0001$). The area under the receiver–operator curve (AUROC) value of the CAP for diagnosing significant steatosis was 0.878 (95% confidence interval, 0.818–0.939), and the optimal CAP cut-off value for detecting significant steatosis was 232.5 db/m. In multivariate analysis, the CAP ($P = 0.0002$) and the liver to spleen ratio ($P = 0.004$) were found to be significantly associated with significant steatosis.

Conclusion: The CAP is a promising tool for rapidly and non-invasively diagnosing steatosis.

Key words: controlled attenuation parameter, FibroScan, liver steatosis, non-invasively diagnose

INTRODUCTION

THE INCIDENCE OF obesity has markedly increased in developed countries in the past few decades. Due to the Westernization of lifestyles in Japan, the frequency of patients presenting with non-alcoholic fatty liver disease (NAFLD) has gradually increased, and NAFLD/non-alcoholic steatohepatitis (NASH) is estimated to affect 10 million people in the general population.[1,2] NAFLD is one of the clinical consequences of obesity and can progress to NASH, ultimately leading to cirrhosis, hepatocellular carcinoma and end-stage liver failure.[3,4]

Liver steatosis is considered to be a risk factor for treatment failure among patients with chronic viral hepatitis, such as that caused by hepatitis B virus (HBV) or hepatitis C virus (HCV).[5] In addition, previous studies demonstrated that the frequency of liver steatosis was significantly lower in hepatitis C patients who

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achieved a sustained virological response (SVR).[6–11] Although the incidence of liver transplantation for end-stage liver disease is increasing, there is a shortage of organs for living donor liver transplantation.[12,13] Accordingly, it is important to properly estimate the degree of liver steatosis in potential donor livers in order to ensure the success of liver transplantation and donor safety.

Liver biopsy is the current gold standard for evaluating steatosis and other histological lesions;[3,4,14] however, it is invasive, subject to sampling error and is sometimes painful.[15,16] To avoid unnecessary biopsy examinations, various non-invasive methods have been developed for the assessment of hepatic steatosis.[17] As fat affects ultrasound propagation, a novel attenuation parameter has been developed to detect and quantify steatosis. This parameter, which is called the controlled attenuation parameter (CAP) because it specifically targets the liver, is based on the ultrasonic properties of the reflected radio frequency signals acquired by the FibroScan M probe (Echosens, Paris, France). Although many reports have demonstrated the utility of the CAP to determine the extent of a patient's liver steatosis,[18–20] its utility for assessing chronic liver disease in Japanese patients is unknown.

The primary objective of our study was to validate the ability of the CAP to detect and quantify steatosis. The secondary objective was to determine whether steatosis could be assessed simultaneously using the FibroScan M probe in patients with biopsy-proven chronic hepatitis due to any cause.

METHODS

Study population

ONE HUNDRED AND fifty-five consecutive patients with suspected chronic hepatitis due to any etiology who underwent liver biopsy and an ultrasound examination with the FibroScan M probe on the same day to calculate their CAP and liver stiffness measurement (LSM) values were enrolled. The patients were recruited at our institution between April and December 2012.

LSM and CAP measurement

After performing conventional ultrasonography to search for hepatocellular carcinoma, the tip of the transducer probe was placed on the patient's skin between the ribs over the right lobe of the liver with the patient lying in the dorsal decubitus position. All patients had their CAP measured using a standard 3.5-MHz M probe. In a preliminary retrospective study, in which the CAP was assessed in 115 patients with chronic liver disease due to various etiologies, the CAP performed well during the detection and semiquantification of steatosis.[18] The LSM was determined using a FibroScan M probe, a Vibration-Controlled Transient Elastography (VCTE; Echosens) device that is designed to measure liver stiffness. Briefly, the VCTE system generates a 50-Hz shear wave that is longitudinally polarized along the ultrasound axis.[21,22] The median value of 10 measurements performed at depths ranging 25–65 mm was adopted as the final liver stiffness value and was expressed in kPa. Only results derived from five valid shots and displaying an interquartile range (IQR)/median liver stiffness ratio of less than 30% were included. The CAP was designed to measure liver ultrasonic attenuation (along the go and return path) at 3.5 MHz using the signals acquired by the FibroScan M probe.[18] The CAP uses a sophisticated guidance process based on VCTE. In brief, the CAP is based on validated measurements, which are subject to the same criteria as the LSM and are obtained from the same signals. Therefore, the LSM and CAP were obtained simultaneously and in the same volume of liver parenchyma (i.e. at depths of between 25 and 65 mm). The median of the individual CAP values was used as the final CAP value, which was expressed in dB/m. The ratio of the IQR of the CAP values to the median CAP value (IQR/Mcap) was calculated as an indicator of variability.[18–20]

Clinical and laboratory evaluations

Biological and clinical parameters were assessed during liver biopsy. The following data were recorded: age; sex; etiology; height; bodyweight; body mass index (BMI); aspartate aminotransferase, alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), total bilirubin, albumin, triglyceride, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, fasting glucose (FBS) and hemoglobin A1c (HbA1c) levels; prothrombin time; and platelet count. All blood sample analyses were performed in our hospital laboratory. Liver density was assessed using the ratio of the mean computed tomography (CT) attenuation value of the liver (in Hounsfield units; HU) to that of the spleen (L/S ratio), which was evaluated using abdominal CT.

Liver biopsy

Liver biopsy was performed by senior surgeons using a 1.2 mm/1.6 mm diameter Menghini needle (Surecut needle, Create Medic Co. Ltd, Japan). The liver specimens measured more than 20 mm in length and were fixed, embedded in paraffin, and then stained with hematoxylin and Masson-trichrome. One experienced pathologist analyzed all of the biopsies independently without knowledge of the clinical data. Steatosis was graded according to the method of Kleiner *et al.*[23] as: S0, steatosis in less than 5% of hepatocytes; S1, 5–33%; S2, 34–66%; and S3, more than 66%

Statistical analyses

The relationships between the CAP and clinical or morphological parameters were evaluated using Spearman's rank correlation coefficient. Multivariate analysis was performed using multiple linear regression to investigate the effects of fibrosis stage, activity grade and steatosis grade on liver stiffness and the CAP. Box plots were used to assess the utility of the non-invasive methods for differentiating between each grade of steatosis. Area under the receiver–operator curve (AUROC) values were computed as well as their 95% confidence intervals (CI) using the Mann–Whitney *U*-test statistic according to the method proposed by Hanley and McNeil.[24] The cut-off value that maximized the accuracy, sensitivity, and negative and positive predictive values of the CAP for diagnosing significant steatosis was computed. All statistical analyses were performed using the SPSS software ver. 18 (SPSS, Chicago, IL, USA). Statistical results associated with *P*-values of less than 0.05 were considered significant.

RESULTS

Patient characteristics

THE BASELINE CHARACTERISTICS of the 155 patients are shown in Table 1. The median age was 55.0 years (range, 24–91), and 92 patients were male. Etiologies of chronic liver diseases were chronic hepatitis B ($n = 17$), chronic hepatitis C ($n = 58$), NASH ($n = 40$), unknown etiology ($n = 35$) and normal liver ($n = 5$). Their median BMI was 24.4 kg/m^2 (range, 15.4–39.2). The patients' median CAP value was 231.0 dB/m (range, 100–400) and their median LSM was 10.7 kPa (range, 2.60–75.0). CT examinations were available in 97 patients, and the median L/S ratio of these patients was 1.05 (range, −0.144 to 2.03).

CAP values for steatosis assessment

The CAP values of each steatosis grade are shown in Figure 1. The median (25–75% quartiles) CAP values for each steatosis grade were: 202.1 dB/m (range, 100–298) for S0, 279.5 dB/m (range, 179–400) for S1, 297.7 dB/m (range, 162–367) for S2 and 323.0 dB/m (range, 290–345) for S3. There were significant differences between the CAP values for S0 and S1 ($P < 0.0001$), S0 and S2 ($P < 0.0001$), and S0 and S3 ($P < 0.0001$). A box plot of the CAP values of the patients with mild (steatosis affecting <5% of hepatocytes) and significant (steatosis affecting ≥5% of hepatocytes) hepatic steatosis is shown in Figure 2. The median CAP value for mild hepatic steatosis was 202.1 dB/m, and that for significant hepatic steatosis was 285.1 dB/m. There was a significant difference between the CAP values for mild and significant hepatic steatosis ($P < 0.0001$).

The AUROC of the CAP for differentiating between mild and significant steatosis is shown in Figure 3. The

Table 1 Bioclinical and historical characteristics of the patients

Characteristics	Patient data
No. of patients	155
Age (years)	55.0 (24–91)
Sex (male/female)	92/63
Etiology (B/C/NASH/others)	17/58/40/40
Height (m)	1.61 (1.40–1.79)
Bodyweight (kg)	64.0 (39.5–117.2)
Body mass index (kg/m^2)	24.4 (15.4–39.2)
AST (IU/L)	52.0 (14–467)
ALT (IU/L)	64.2 (7–657)
Total bilirubin (mg/dL)	1.0 (0.3–9.3)
Serum albumin (g/dL)	4.2 (2.8–5.4)
Prothrombin (%)	93.7 (43–140)
Platelet count (×104/μL)	19.3 (6.2–54.3)
Triglycerides (mg/dL)	113.5 (23–479)
Total cholesterol (mg/dL)	182.9 (68–336)
High-density lipoprotein cholesterol (mg/dL)	60.5 (12–179)
Low-density lipoprotein cholesterol (mg/dL)	113.6 (26–204)
Fasting blood sugar (mg/dL)	108.9 (21–179)
HbA1c (NGSP, %)	6.0 (4.8–10.1)
Controlled attenuation parameter (CAP, dB/m)	231.0 (100–400)
Liver stiffness measurements (LSM, kPa)	10.7 (2.60–75.0)
L/S ratio	1.05 (−0.144 to 2.03)

All data are median (range).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; B, HBs antigen positive; HbA1c, hemoglobin A1c; C, HCV antibody positive; L/S, liver/spleen; NASH, non-alcoholic steatohepatitis; NGSP, National Glycohemoglobin Standardization Program.

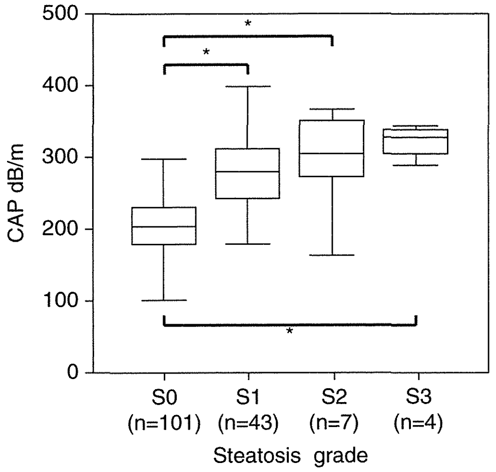


Figure 1 Distribution of controlled attenuation parameter (CAP) for each steatosis grade. The bottom and top of each box represent the 25th and 75th percentiles, giving the interquartile range. The line through the box indicates the median, and the error bars indicate the 10th and 90th percentiles. $*P < 0.0001$.

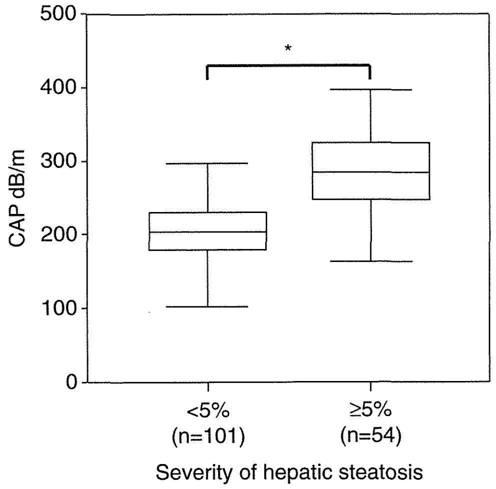
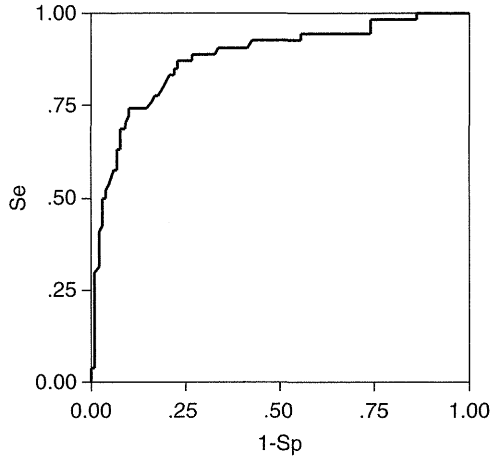


Figure 2 Box plot of controlled attenuation parameter (CAP) in hepatic steatosis according to severity <5% and ≥5%. There is significant correlation between CAP and frequency of steatosis. $*P < 0.0001$.



AUROC (95%CI)	0.878 (0.818-0.939)
Cut-off	232.5
Se/Sp	87.0%/77.2%
PPV/NPV	75.2%/87.0%

Figure 3 AUROC to compare the diagnostic accuracy of liver steatosis (<5% and ≥5%) assessed by controlled attenuation parameter. AUROC, area under the receiver–operator curve; NPV, negative predictive value; PPV, positive predictive value; Se, sensitivity; Sp, specificity.

CAP displayed an AUROC value of 0.878 (95% CI, 0.818–0.939) for diagnosing significant hepatic steatosis. The optimal CAP cut-off value for differentiating between mild and significant hepatic steatosis was 232.5 dB/m, which produced sensitivity and specificity values of 87.0% and 77.2%, respectively, as well as a positive predictive value (PPV) of 75.2% and a negative predictive value (NPV) of 87.0%. The AUROC based on the individual etiologies were shown in Supporting Information Figure S1.

The results of our univariate analysis of the factors associated with significant steatosis are shown in Table 2. Among the analyzed factors, BMI, cholinesterase, the CAP value and L/S ratio displayed the most significant associations with significant steatosis ($P < 0.0001$). ALT ($P = 0.0001$), triglyceride ($P = 0.002$), HbA1c ($P = 0.002$), alkaline phosphatase ($P = 0.007$), white blood cell ($P = 0.020$), platelet count ($P = 0.020$), GGT ($P = 0.028$), FBS ($P = 0.036$) and total cholesterol ($P = 0.043$) also displayed significant associations with significant hepatic steatosis. In the multivariate analysis, only the CAP value (odds ratio, 27.656; 95% CI, 4.762–160.622; $P = 0.0002$) and L/S ratio (odds ratio, 10.881; 95% CI, 2.101–56.361; $P = 0.004$) were significantly associated with significant steatosis (Table 3).

DISCUSSION

IN JAPAN, MUCH attention has been paid to HBV/HCV-infected patients over the past few decades because there are high numbers of carriers of these viruses in Japan, and most cases of cirrhosis and hepa-

Table 2 Factors associated with steatosis ≥5% on liver biopsy (univariate analysis)

Variable		Severity of hepatic steatosis				*P*-value
		<5%		≥5%		
		n	Mean ± SD	*n*	Mean ± SD	
Age	<60/≥60	50/51	45.8 ± 9.8/66.6 ± 6.1	34/20	44.7 ± 10.3/65.6 ± 4.8	0.129
Sex	Female/male	44/57		19/35		0.391
BMI (kg/m^2)	<25/≥25	74/27	21.6 ± 2.0/26.8 ± 1.9	18/36	23.1 ± 2.0/29.3 ± 4.0	<0.0001
AST (IU/L)	<33/≥33	50/51	25.1 ± 5.0/90.9 ± 99.5	19/35	24.1 ± 5.6/49.2 ± 17.7	1.000
ALT (IU/L)	<35/≥35	51/50	24.0 ± 7.0/113.8 ± 114.0	12/42	24.9 ± 8.0/65.0 ± 22.7	0.0001
ALP (IU/L)	<359/≥359	68/33	223 ± 60/587 ± 243	47/7	222 ± 55/525 ± 189	0.007
GGT (IU/L)	<41/≥41	53/48	23.4 ± 8.6/281 ± 522	18/36	26.0 ± 9.6/103 ± 73	0.028
Cholinesterase (IU/L)	<300/≥300	69/32	224 ± 58/339 ± 35	16/38	228 ± 56/381 ± 50	<0.0001
Total bilirubin (mg/dL)	<1.2/≥1.2	83/18	0.74 ± 0.19/2.48 ± 1.9	47/7	0.73 ± 0.19/1.51 ± 0.38	0.498
Serum albumin (mg/dL)	<4.3/≥4.3	51/50	3.73 ± 0.39/4.50 ± 0.26	24/30	3.97 ± 0.26/4.64 ± 0.31	0.503
Prothrombin (%)	<70/≥70	5/96	59.8 ± 10.5/94.7 ± 12.8	2/52	57.5 ± 5.0/96.4 ± 10.9	1.000
White blood cell (/μL)	<4000/≥4000	25/76	3318 ± 578/6143 ± 1806	5/49	3522 ± 485/6929 ± 2836	0.020
Platelet count (/μL)	<22×10^4/≥22×10^4	75/26	15.4 ± 4.1/27.4 ± 5.2	30/24	15.4 ± 4.1/27.4 ± 6.6	0.020
Triglyceride (mg/dL)	<149/≥149	81/13	85.9 ± 28.7/182 ± 46.5	32/19	82.5 ± 29.7/236 ± 83.8	0.002
Total cholesterol (mg/dL)	<179/≥179	56/45	149 ± 25/213 ± 33	20/34	162 ± 15/212 ± 22	0.043
FBS (mg/dL)	<109/≥109	65/33	97.7 ± 6.6/141.8 ± 37.0	25/27	99.3 ± 6.7/133.9 ± 32.0	0.036
HbA1c (NGSP, %)	<6.2/≥6.2	63/14	5.6 ± 3.7/6.5 ± 0.6	26/21	5.2 ± 0.3/6.9 ± 1.3	0.002
CAP (dB/m)	<232.5/≥232.5	76/25	182 ± 34/263 ± 31	7/47	196 ± 24/298 ± 41	<0.0001
LSM (kPa)	<10.7/≥10.7	77/24	6.0 ± 1.8/29.0 ± 22.0	36/18	6.1 ± 2.1/15.5 ± 4.6	0.255
L/S ratio	≥1.1/ < 1.1	40/11	1.27 ± 0.16/1.01 ± 0.07	13/33	1.18 ± 0.08/0.74 ± 0.29	<0.0001

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; CAP, controlled attenuation parameter; GGT, γ-glutamyltransferase; HbA1c, hemoglobin A1c; L/S, liver/spleen; LSM, liver stiffness measurement; NGSP, National Glycohemoglobin Standardization Program; SD, standard deviation.

tocellular carcinoma in Japan are associated with persistent HBV or HCV infection.[25] In recent years, NAFLD has become a major social problem in Japan due to the Westernization of lifestyles and the increasing rates of obesity and diabetes.[26] Approximately 30% of NAFLD patients are considered to progress to NASH, a more severe form of NAFLD, which leads to more advanced fibrosis and ultimately cirrhosis.[27] Among chronic viral hepatitis patients, liver steatosis is a risk factor for infection and treatment failure.[5] Chronic HCV infection is associated with fatty liver changes, and HCV patients display a higher incidence of fatty changes than patients with other chronic liver dysfunctions.[28,29] Furthermore, Okanoue *et al.* demonstrated that the frequency of liver steatosis was significantly lower in hepatitis C patients who achieved an SVR.[11] Therefore, it is important to diagnose and evaluate the severity of steatosis to improve its treatment and prognosis. Liver biopsy is the current gold standard for evaluating steatosis and other histological lesions;[3,4,14] however, liver biopsy can be affected by sampling error,[15,16] is an invasive and often painful procedure, and can result in severe complications.[30,31] Moreover, the repetition of liver biopsy to monitor changes in steatosis is difficult. In light of these obstacles, various non-invasive methods have been developed for the assessment of hepatic histology, particularly fibrosis.[17,32] Steatosis can also be diagnosed by non-invasive means and is mainly diagnosed using conventional imaging techniques, for example, CT, multiple resonance imaging (MRI), magnetic resonance

Table 3 Factors associated with steatosis ≥5% on liver biopsy (multivariate analysis)

Variable	Odds ratio	95% confidence interval	*P*-value
CAP ≥232.5 (dB/m)	27.656	4.762–160.622	0.0002
L/S ratio <1.1	10.881	2.101–56.361	0.004

Factors: body mass index, ≥25; alanine aminotransferase, ≥35; alkaline phosphatase, ≥359; γ-glutamyltransferase, ≥41; cholinesterase, ≥300; white blood cell, ≥4000; platelet count, ≥20 × 10^4; triglyceride, ≥149; total cholesterol, ≥179; fasting blood sugar, ≥109; hemoglobin A1c, ≥5.7; controlled attenuation parameter (CAP), ≥243.5; liver/spleen (L/S) ratio <1.1.

spectroscopy or ultrasonography, with the latter being the most commonly used method.[33,34] However, these techniques suffer from various pitfalls; namely, they are costly, not easily available, operator-dependent and/or display poor sensitivity.[32,34,35] Moreover, existing methods cannot simultaneously assess hepatic fibrosis and steatosis. To overcome these limitations, the CAP, which was designed to produce immediate results and be reproducible and operator- and device-independent, was developed.[36] Previous studies have shown the utility of the CAP for assessing the severity of steatosis.[18–21,23,37,38]

In our study, we have demonstrated that the CAP is correlated with steatosis grade and can be used to non-invasively identify steatosis in Japanese patients. The AUROC of the CAP for detecting significant steatosis ($\geq$5% of hepatocytes affected) was 0.878 (95% CI, 0.818–0.939), and a CAP threshold of 232.5 dB/m demonstrated 87.0% sensitivity and 77.2% specificity for detecting significant steatosis. This study is the first to report the utility of the CAP in Japanese subjects. A previous study reported similar findings in a study of 153 patients with chronic liver disease due to any etiology, in whom the CAP displayed an AUROC of 0.81 for diagnosing significant stenosis, and a CAP threshold of 283 dB/m demonstrated 76% sensitivity and 79% specificity for significant steatosis.[20] Sasso *et al.* studied 115 patients with various liver disorders. As a result, they found that the CAP displayed an AUROC of 0.91 for detecting significant steatosis, and a CAP threshold of 238 dB/m exhibited 91% sensitivity and 81% specificity for significant steatosis.[18,19] The discrepancies between these studies may be related to differences in the study populations including in their disease etiologies, the prevalence of obesity and the extent of subcutaneous adiposity, the severity of the patients' steatosis and racial differences, all of which could influence CAP performance because of spectrum bias. Further studies in larger cohorts would help to refine the patient data characteristics of the CAP.

In some patients, steatosis can progress to cirrhosis and end-stage liver disease.[39] Furthermore, liver transplantation is the only treatment option for end-stage liver failure. In such cases, it is important to select an appropriate donor in order to achieve good donor and recipient outcomes. The implantation of donor livers with severe fatty infiltration is associated with a high incidence of severe ischemic damage, resulting in primary dysfunction and/or primary non-function after liver transplantation.[40–44] To reduce the risk of progressive liver disease and achieve a successful liver transplantation, it is important to estimate the extent of liver steatosis. A few reports have suggested that there is a risk associated with mild macrovascular steatosis after right hepatectomy in living donors.[45,46] Goldaracena *et al.* reported that the liver pool can be safely expanded using extremely marginal liver grafts. It is considered that steatosis should not affect more than 30% of such grafts;[12] therefore, most centers only accept donor livers from individuals in whom hepatic steatosis affects 20% or less of the liver.[47–49] In this study, we thought that we could detect steatosis more strictly by using a 5% cut-off value according to Kleiner *et al.*[23] Accordingly, we selected 5% as the cut-off value. When we selected a 10% cut-off value, the result was similar (AUROC, 0.878 [95% CI, 0.810–0.947]; CAP threshold, 258.0 dB/m; sensitivity, 81.8%; specificity, 87.4%).

Imaging studies such as ultrasonography, CT and MRI can depict the characteristic features of fatty liver.[30–34,39] In particular, CT has proven to be useful for diagnosing and quantifying liver fat non-invasively. The HU attenuation value of the liver on CT scans is usually higher than that of the spleen. However, the presence of fat in the liver will reduce its HU attenuation value. Thus, an L/S ratio of less than 1.0 can be used to effectively diagnose the presence of liver fat, and studies also have shown that liver HU attenuation values of less than 40 HU represent a liver fat content of more than 30%.[34,39] Furthermore, Oliva *et al.* reported that the use of an L/S ratio of less than 1.2 resulted in all cases of fatty liver being detected, whereas some authors reported cut-off values of 1.0 or 1.1 for fatty liver.[49]

In our study, significant hepatic steatosis was significantly associated with a CAP of 232.5 dB/m or more and an L/S ratio of less than 1.1. These results demonstrate that the CAP accurately predicts the degree of steatosis. Furthermore, the CAP is an easier and cheaper procedure than CT and does not involve radiation exposure.[47,49,50]

This study had several limitations. One limitation was that our study involved a relatively small population, which limited the precision of our results. Second, although a correlation was observed between the degree of steatosis and the CAP ($r = 0.517$, $P < 0.0001$, Pearson product-moment correlation coefficient), our study population was highly selected; namely, it included patients with mild hepatic steatosis, which also limited the precision of our results. Third, our sample size was limited in part because of the difficulty of obtaining valid CAP measurements in obese patients using the FibroScan M probe. Further studies are necessary to develop a CAP algorithm for such patients. Finally, selection bias was another limitation of this study because we did not examine patients who displayed clinical evidence of hepatic decompensation.

In conclusion, the CAP can be used for steatosis detection and semiquantification and possesses several advantages; namely, it is non-invasive, easy to perform, provides immediate results and is inexpensive in comparison with other measurement modalities. Moreover, the CAP can provide an immediate assessment of steatosis and be obtained at the same time as the LSM, which is used to stage hepatic fibrosis. Further studies are necessary to validate our findings in larger cohorts and to define optimal CAP thresholds. If these results are confirmed, the CAP could be useful for the diagnosis of steatosis, not only in chronic liver disease, but also in liver graft evaluations, longitudinal monitoring of disease progression or the response to therapy, population-based epidemiological or observational studies, and drug discovery.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Figure S1 (a) AUROC to compare the diagnostic accuracy of liver steatosis (<5% and ≥5%) assessed by CAP in HBV patients. (b) AUROC to compare the diagnostic accuracy of liver steatosis (<5% and ≥5%) assessed by CAP in HCV patients. (c) AUROC to compare the diagnostic accuracy of liver steatosis (<5% and ≥5%) assessed by CAP in NASH patients. (d) AUROC to compare the diagnostic accuracy of liver steatosis (<5% and ≥5%) assessed by CAP in patients of other etiologies. AUROC, area under the receiver–operator curve; CAP, controlled attenuation parameter; HBV, hepatitis B virus; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; NPV, negative predictive value; PPV, positive predictive value; Se, sensitivity; Sp, specificity.

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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.***

KEY WORDS: HBsAg; histological activity; inflammation; microRNA

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,