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LETTER TO THE EDITOR

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

Masataka Tsuge · Kazuaki Chayama

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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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M. Tsuge · K. Chayama (✉)
Department of Gastroenterology and Metabolism,
Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan
e-mail: chayama@hiroshima-u.ac.jp

M. Tsuge · K. Chayama
Liver Research Project Center,
Hiroshima University, Hiroshima, Japan

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

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

Keiichi Masaki,[1] Shintaro Takaki,[1] Hideyuki Hyogo,[1] Tomoki Kobayashi,[1] Takayuki Fukuhara,[1] Noriaki Naeshiro,[1] Yoji Honda,[1] Takashi Nakahara,[1] Atsushi Ohno,[1] Daisuke Miyaki,[1] Eisuke Murakami,[1] Yuko Nagaoki,[1] Tomokazu Kawaoka,[1] Masataka Tsuge,[1] Nobuhiko Hiraga,[1] Akira Hiramatsu,[1] Michio Imamura,[1] Yoshiiku Kawakami,[1] Hiroshi Aikata,[1] Hidenori Ochi,[1] Shoichi Takahashi,[1] Koji Arihiro[2] and Kazuaki Chayama[1]

Departments of [1]Gastroenterology and Metabolism and [2]Anatomical Pathology, Hiroshima University Hospital, Hiroshima, Japan

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

Correspondence: Shintaro Takaki, Hiroshima University Hospital, Gastroenterology and Metabolism, Hiroshima, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Email: takakiss@hiroshima-u.ac.jp
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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 speci-

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mens 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).

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.

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

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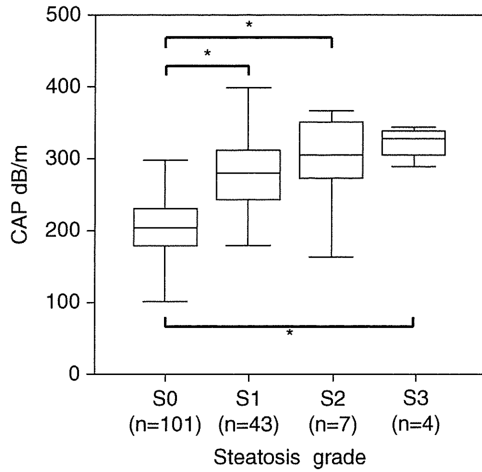
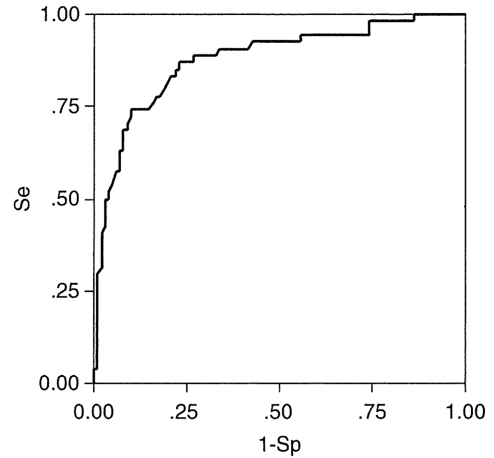


Figure 1 Distribution of controlled attenuation parameter (CAP) for each steatosis grade. The bottom and top of each box represent the 25th and 75th percentiles, giving the interquartile range. The line through the box indicates the median, and the error bars indicate the 10th and 90th percentiles. $^{*}P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



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.

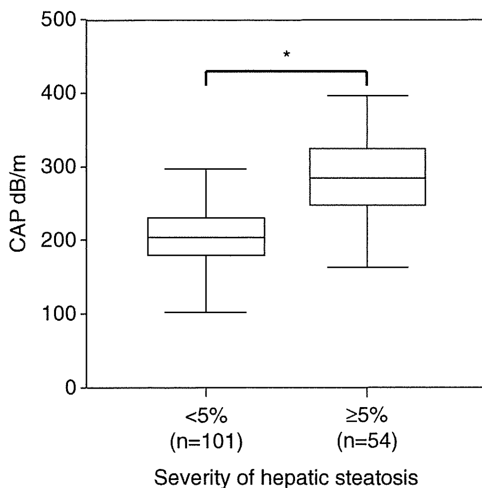


Figure 2 Box plot of controlled attenuation parameter (CAP) in hepatic steatosis according to severity <5% and ≥5%. There is significant correlation between CAP and frequency of steatosis. $^{*}P < 0.0001$.

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-

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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/m²)	<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)	<22×10⁴/≥22×10⁴	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 × 10⁴; 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.

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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 (≥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

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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

Keiko Arataki,[1,2,3,4] C. Nelson Hayes,[1,2,3] Sakura Akamatsu,[1,2,3] Rie Akiyama,[1,2,3] Hiromi Abe,[1,2] Masataka Tsuge,[3,5] Daiki Miki,[1,2,3] Hidenori Ochi,[1,2,3] Nobuhiko Hiraga,[1,2,3] Michio Imamura,[1,2,3] Shoichi Takahashi,[1,2] Hiroshi Aikata,[1,3] Tomokazu Kawaoka,[1,2,3] Hiroiku Kawakami,[1,2,3] Waka Ohishi,[3,6] and Kazuaki Chayama[1,2,3]*

[1]*Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan*
[2]*Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN, Hiroshima, Japan*
[3]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan*
[4]*Tsuchiya General Hospital, Hiroshima, Japan*
[5]*Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan*
[6]*Department of Clinical Studies, Radiation Effects Research Foundation, Hiroshima, Japan*

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

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KEY WORDS: HBsAg; histological activity; inflammation; microRNA

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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*Correspondence to: Kazuaki Chayama, M.D., Ph.D., Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-Ku, Hiroshima 734-8551, Japan. E-mail: chayama@hiroshima-u.ac.jp

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INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Study Patients

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

Viral Markers

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

MicroRNA Analysis

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

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

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

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

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

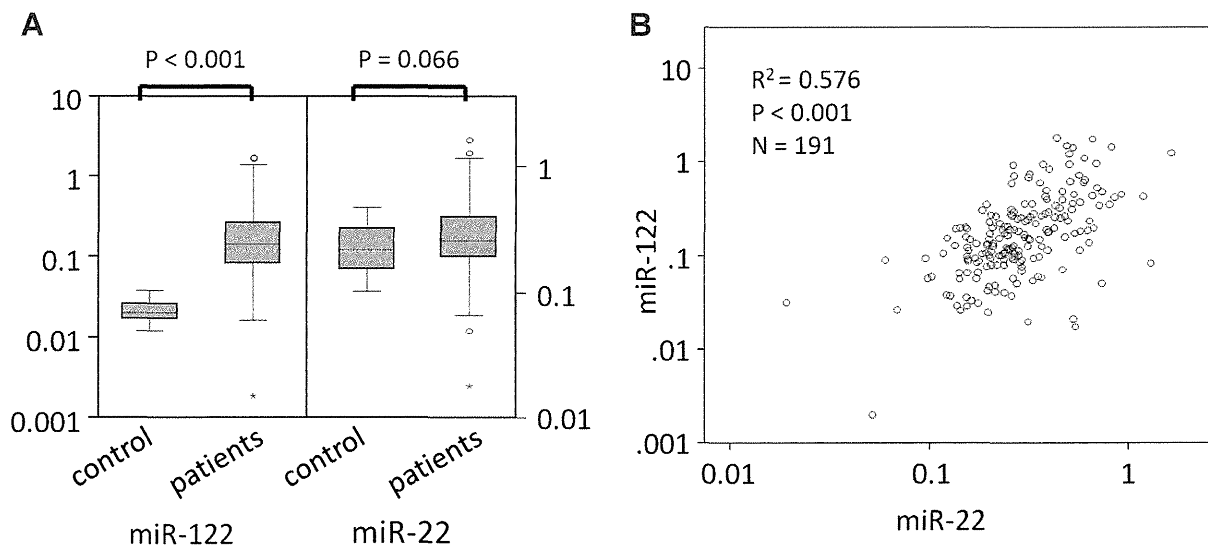

Fig. 1. Detection of miR-122 and miR-22 in patients infected with HBV and in healthy subjects and the relationship between miR-122 and miR-22. **A**: Serum levels of miR-122 and miR-22 in patients infected with HBV (171) and in healthy controls (22). Boxes represent 25–75 percentiles, and horizontal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

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

Statistical Analysis

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

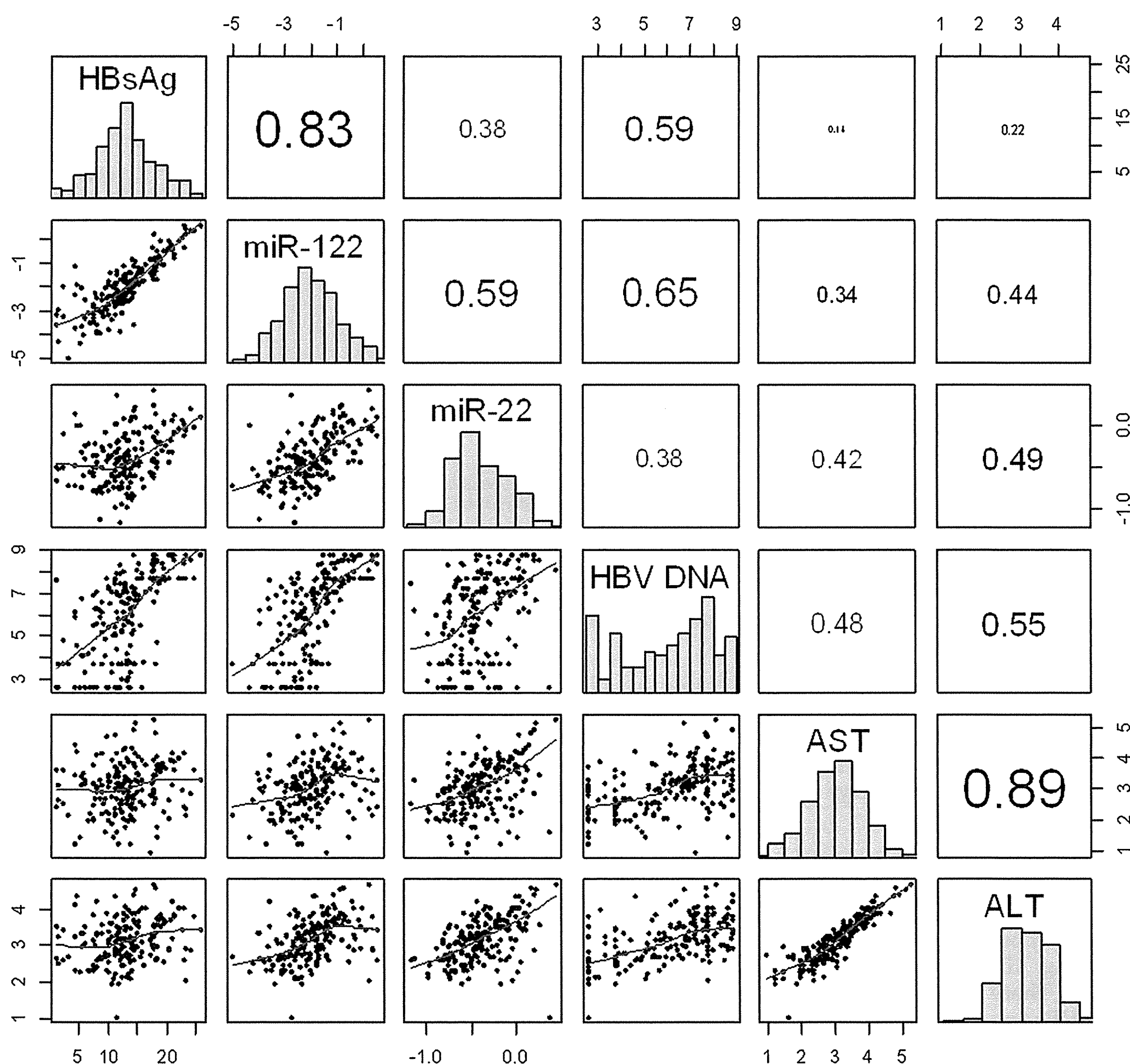

Fig. 2. Pairwise correlations of miR-122 and miR-22 with HBsAg, HBV DNA, ALT, and AST levels. Serum levels of miR-122 and miR-22 were compared with serum HBsAg and HBV DNA titers and with ALT and AST levels using the Spearman rank correlation coefficient.

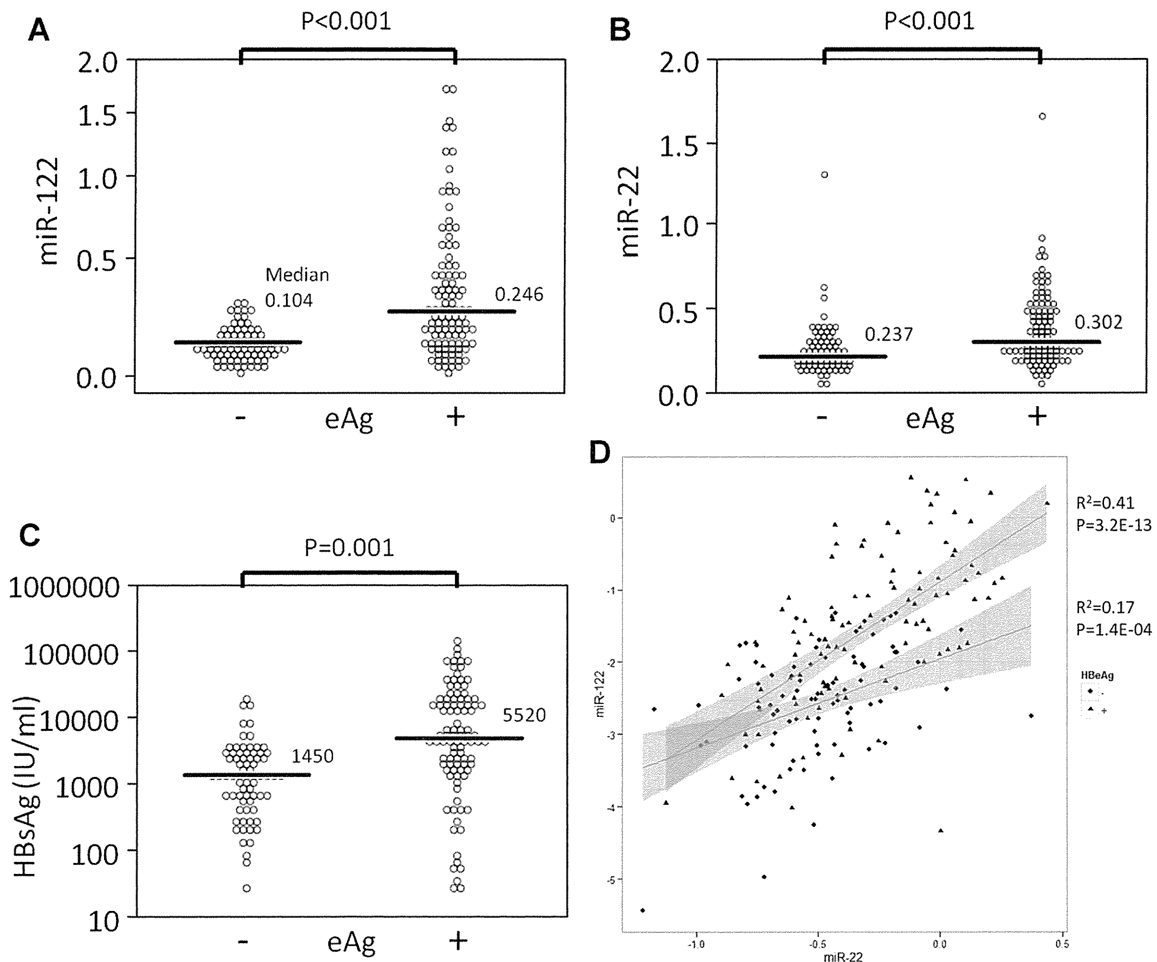

Fig. 3. Comparison of miR-122 and miR-22 with HBsAg levels between patients positive or negative for HBeAg. Serum levels of miR-122 (**A**), miR-22 (**B**), and HBsAg (**C**) were analyzed using the Mann–Whitney U test. Bars indicate median values.

RESULTS

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

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

miR-122 and miR-22 Levels and Viral Markers

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

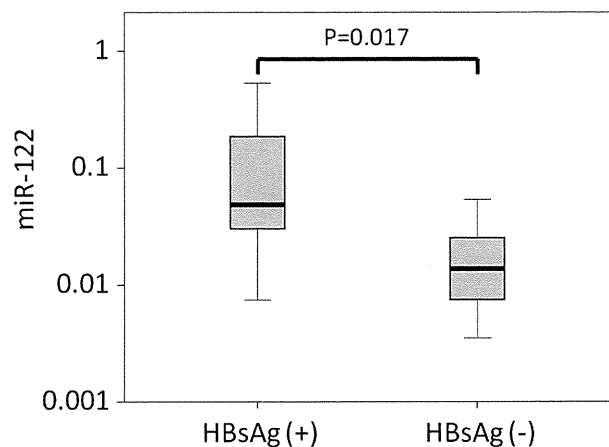

Fig. 4. miR-122 and HBsAg elimination. miR-122 levels before and after HBsAg elimination are shown for patients who became negative for HBsAg (n = 13). Bars represent median, minimum, and maximum levels, and boxes represent the 25th and 75th percentiles. Data were analyzed using the Mann–Whitney U test.

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

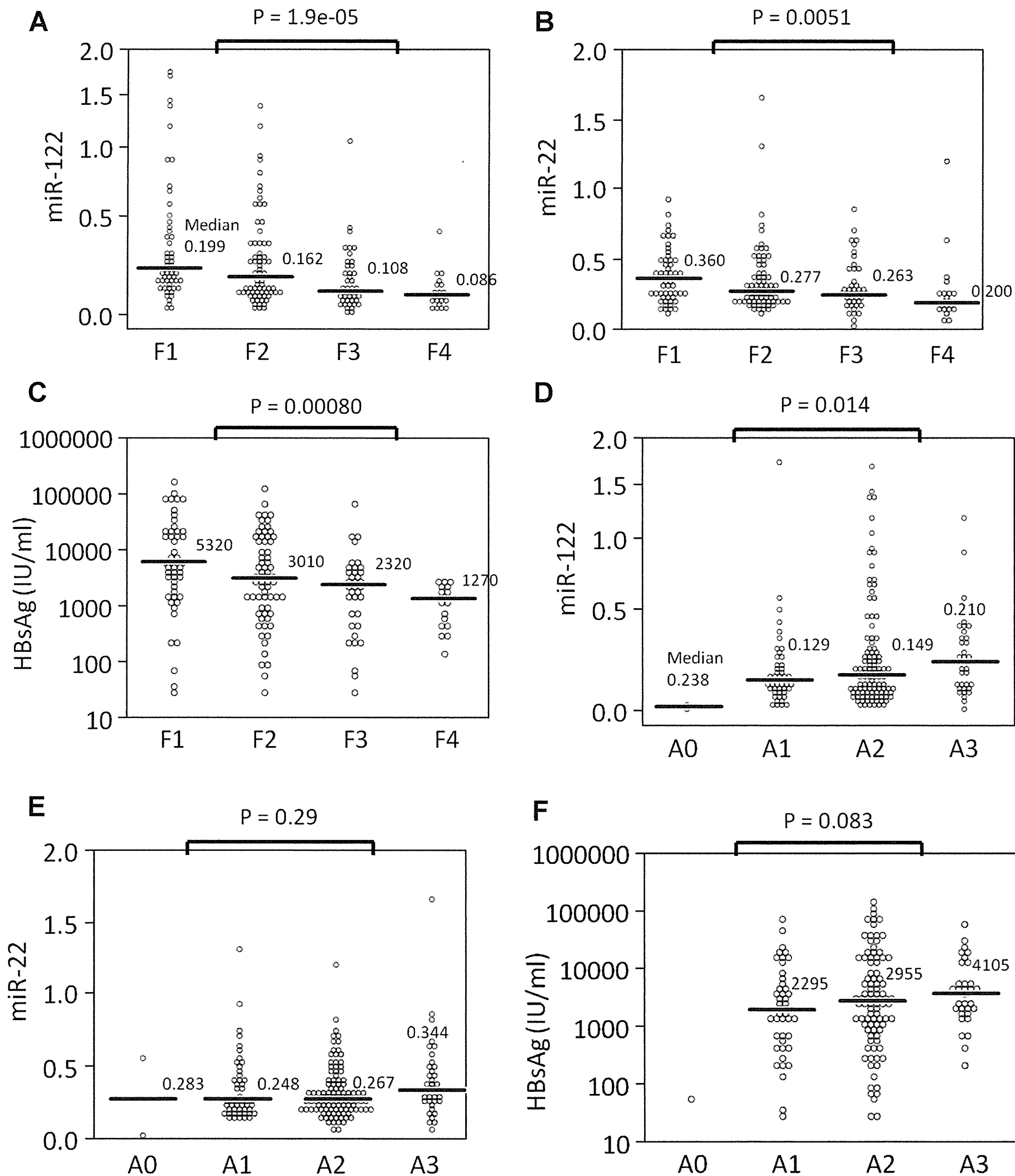

Fig. 5. Stage of fibrosis and histological inflammation activity by liver biopsy and miR-122, miR-22, and HBsAg levels. Serum levels of miR-122, miR-22, and HBsAg were plotted according to the stage of fibrosis (**A**, **B**, and **C**, respectively) and inflammation activity (**D**, **E**, and **F**).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

miR-122 Levels in Patients Who Became Negative for HBsAg

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

miR-122 and miR-22 Levels and Histological Findings

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

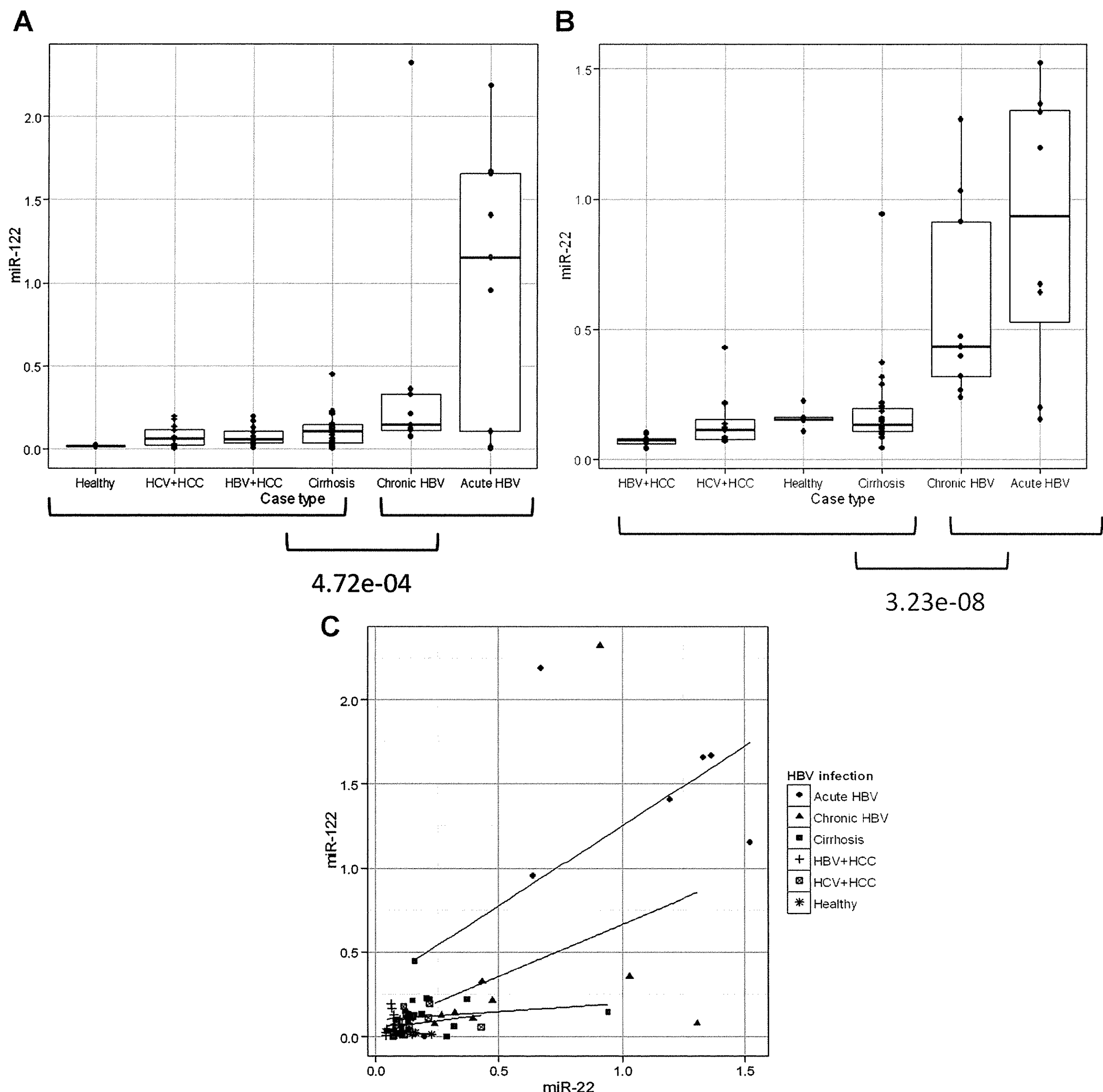

Fig. 6. miR-122 and miR-22 levels by case type. To examine the association of serum miR-122 and miR-22 with HBV infection, expression levels of miR-122 (**A**) and miR-22 (**B**) were compared among healthy controls, patients with acute or chronic HBV infection, liver cirrhosis, and HCC associated with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

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

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

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

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