were incubated with IFN-β (1,000 IU/ml) for up to 72 h for determining the expression levels of miR-195. Control indicates non-treated cells. *$P < 0.05$, **$P < 0.01$ compared with control. (B – D) LX-2 cells were transfected with 50 nM miR-195 precursor or a negative control (control). (B) mRNA expression levels of E2F3, CDK4, CDK6, cyclin D1, cyclin E1, and p21 measured at 24, 48, 72, and 96 h posttransfection. (C) Protein expression of E2F3, CDK4, CDK6, cyclin D1, cyclin E1, and p21 examined at 48, 72, and 96 h posttransfection. (D) Growth of LX-2 cells transfected with miR-195 or untreated control was measured using a WST-1 assay. *$P < 0.05$, **$P < 0.01$ compared with control.

Fig. 5. Interaction of miR-195 with the 3'UTR of cyclin E1 mRNA. (A) Schematic indication of the putative miR-195 target sites in the 3'UTR of the cyclin E1 mRNA. Tested sequences indicate the regions that were inserted into the luciferase reporter vector. (B) Predicted pairing of the target region and miRNAs. (C) Structure of the luciferase reporter vector [14]. The putative miR-195 target region in cyclin E1 3'UTR (tested sequence) was ligated into the MCS. Arrows indicate the gene directions. Amp^R indicates an ampicillin resistance gene. (D) Reporter gene assay of the interaction between the 3'UTR of cyclin E1 mRNA and miR-195 in LX-2 cells. Results are expressed as the relative activities against the activity in the presence of the control. *$P < 0.05$, **$P < 0.01$ compared with control.

Fig. 6. Regulation of cyclin E1 expression by IFN-β and miR-195. LX-2 cells were transfected with 50 nM miR-195 inhibitor or a negative control. After 6 h, the culture

medium was changed and then IFN-β (1,000 IU/ml) was added. Cells were then incubated for the indicated time periods. (A) mRNA expression levels of cyclin E1. (B) Protein expression levels of cyclin E1. GAPDH are for loading adjustment. Control; cells were transfected with a negative control and incubated without IFN-β. $*P < 0.05$, $**P < 0.01$.

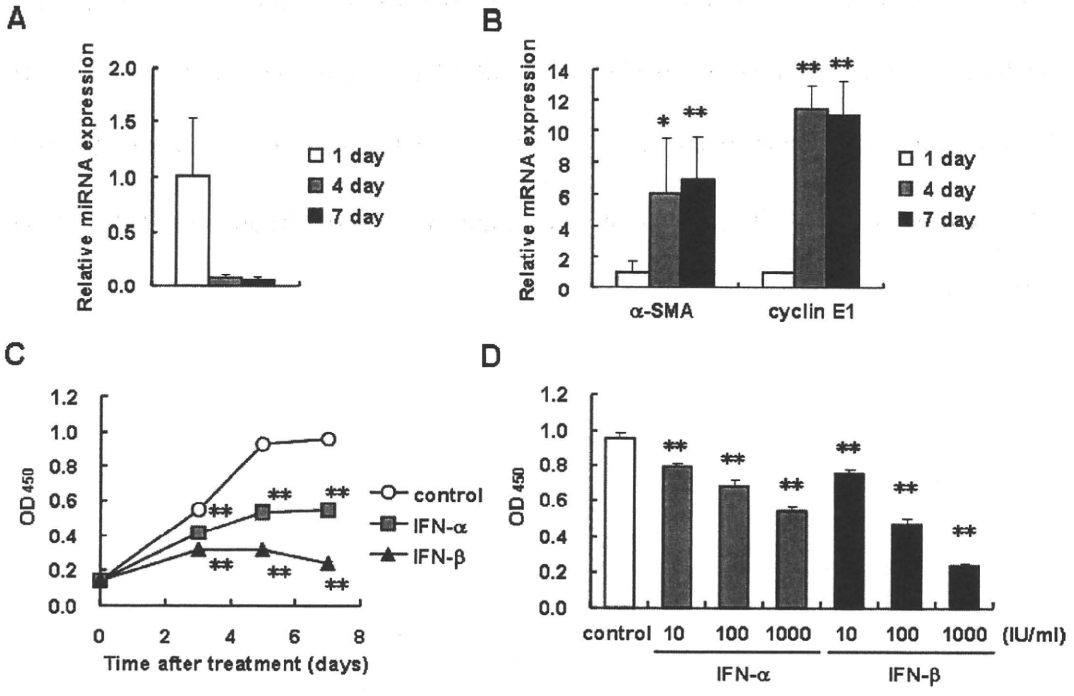

Fig. 1

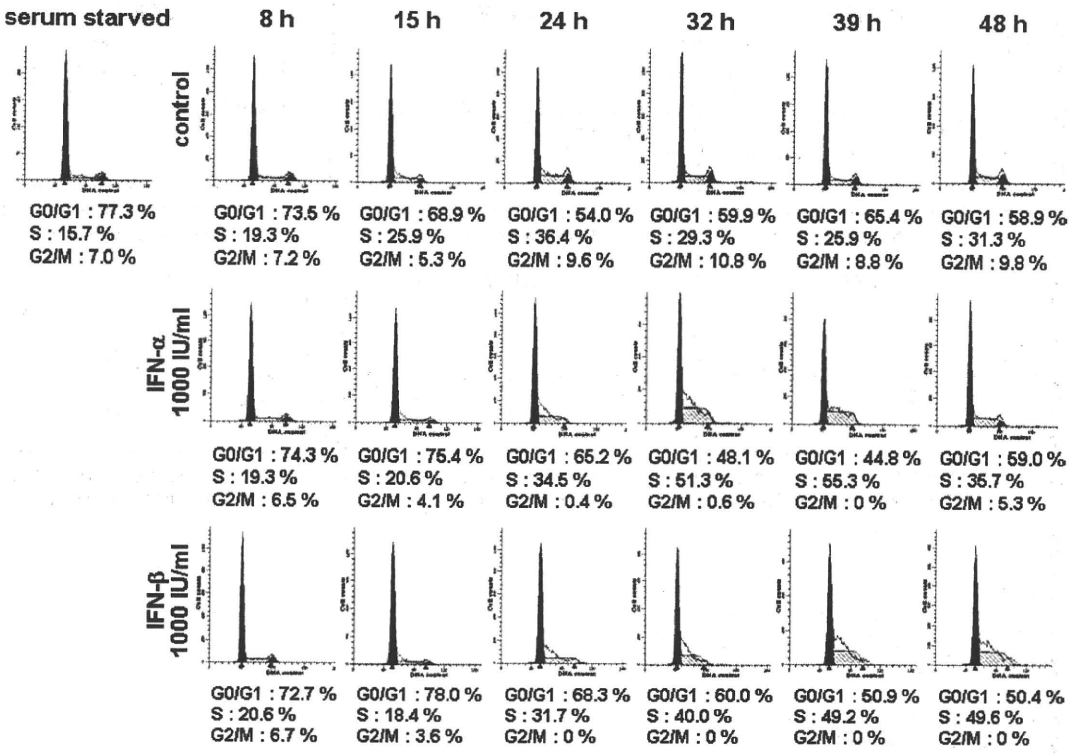

Fig. 2

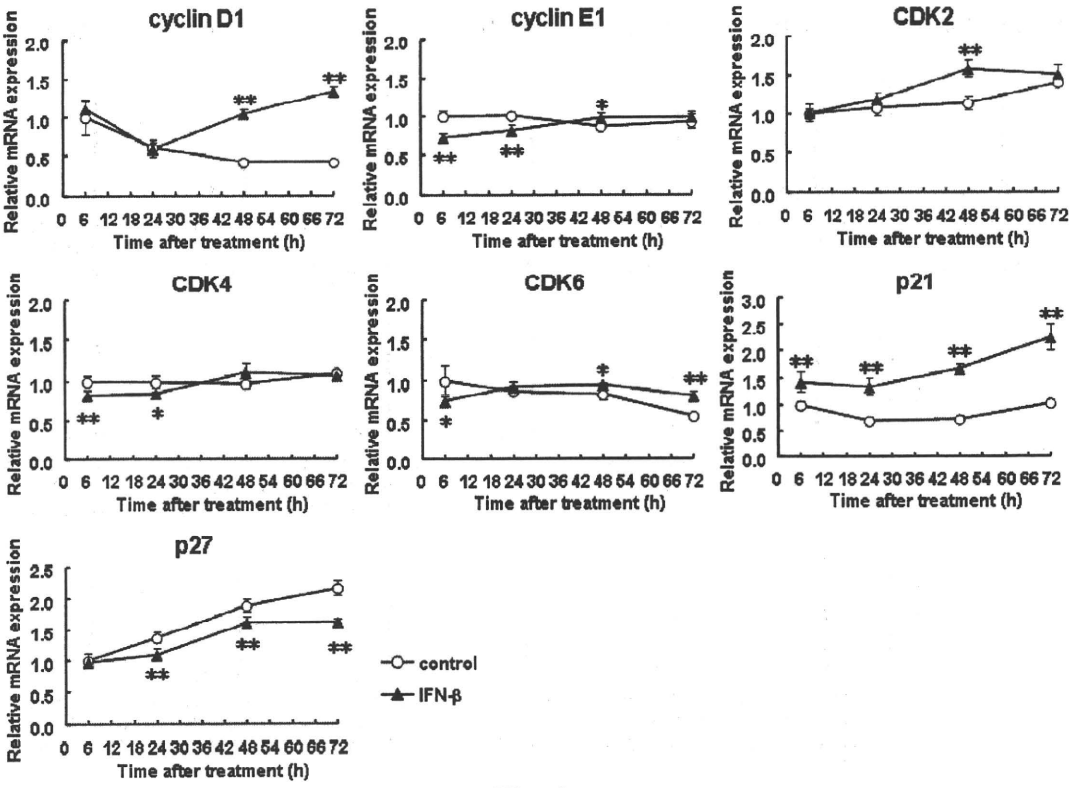

Fig. 3

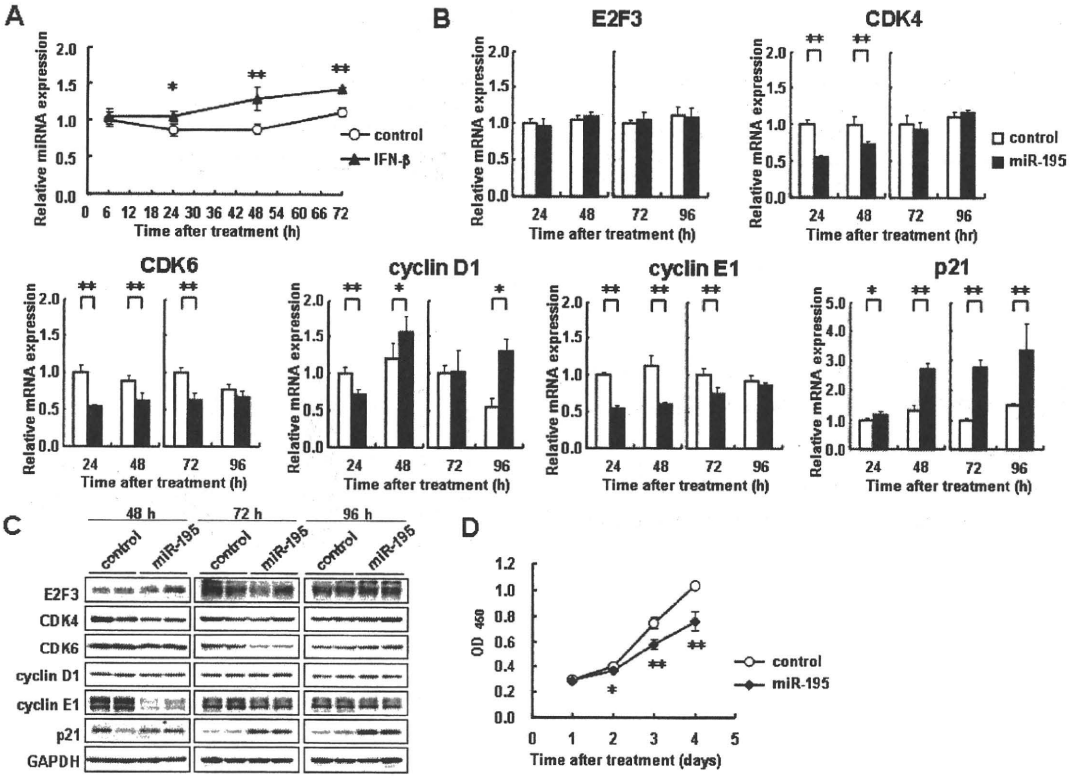

Fig. 4

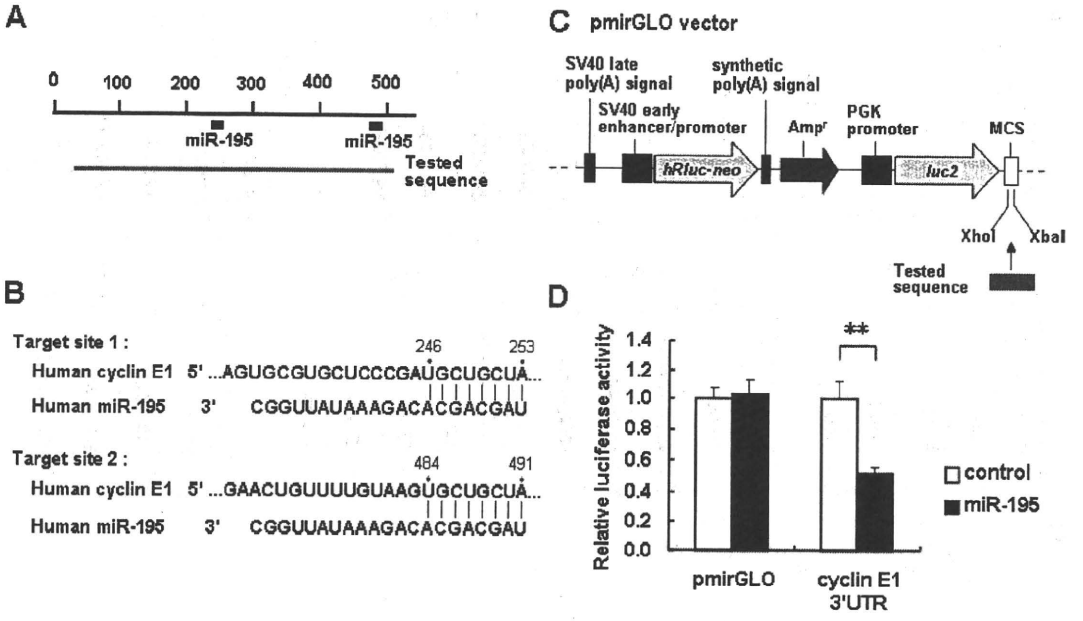

Fig. 5

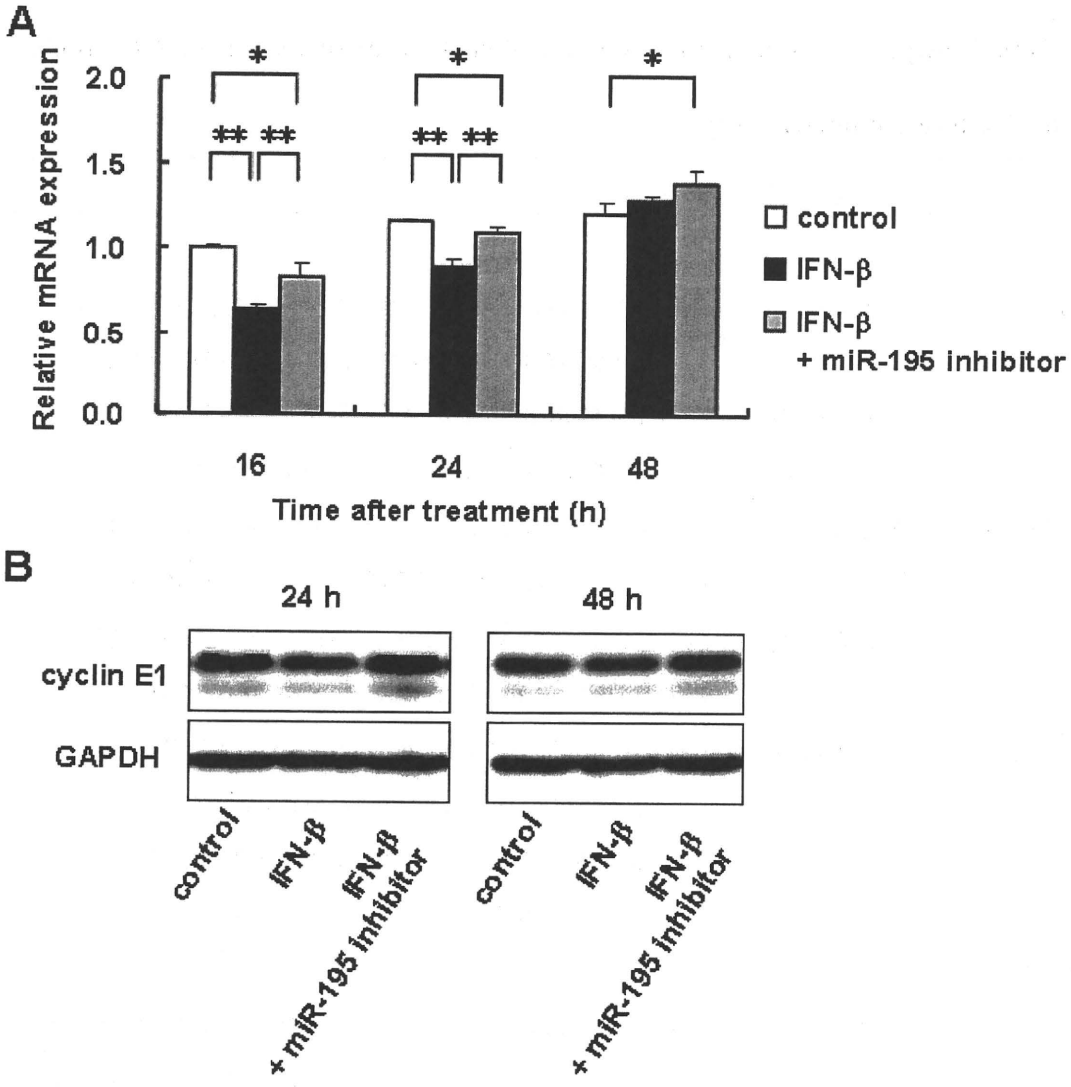

Fig. 6

Table 1. Sequences of primers used in real-time PCR analyses and 3'UTR cloning for luciferase reporter assay

Gene	Accession No.	Sequence
real-time PCR		
CDK6	NM_001259	Forward: 5'-ATATCTGCCTACAGTGCCCTGTCTC-3'
		Reverse: 5'-GTGGGAATCCAGGTTTTCTTTGCAC-3'
Cyclin E1	NM_001238	Forward: 5'-GCAGTATCCCCAGCAAATC-3'
		Reverse: 5'-TCAAGGCAGTCAACATCCA-3'
Cyclin D1	NM_053056	Forward: 5'-GCTGTGCATCTACACCGACAACTC-3'
		Reverse: 5'-AGGTTCCACTTGAGCTTGTTCACC-3'
E2F3	NM_001949	Forward: 5'-CCAACTCAGGACATAGCGATTGCTC-3'
		Reverse: 5'-AGGAATTTGGTCCTCAGTCTGCTGT-3'
GAPDH	NM_002046	Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'
		Reverse: 5'-TGGTGAAGACGCCAGTGGA-3'
p21	NM_000389	Forward: 5'-AGCAGAGGAAGACCATGTGGA-3'
		Reverse: 5'-GGAGTGGTAGAAATCTGTCATGCT-3'
3'UTR cloning		
Cyclin E1	NM_001238	Forward: 5'-TTCTCGAGATCCTTCTCCACCAAAGACAGTT-3'
		Reverse: 5'-TTTCTAGAGAATGGATAGATATAGCAGCACTTACA-3'

The forward and reverse primers for 3'UTR cloning carried the XhoI and XbaI sites at their 5'-ends, respectively.

Hepatology Research

Hepatology Research 2010; **40**: 853–861 doi: 10.1111/j.1872-034X.2010.00687.x

Original Article

Usefulness of transient elastography for assessment of liver fibrosis in chronic hepatitis B: Regression of liver stiffness during entecavir therapy

Masaru Enomoto,[1] Mami Mori,[1] Tomohiro Ogawa,[1] Hideki Fujii,[1] Sawako Kobayashi,[1] Shuji Iwai,[1] Hiroyasu Morikawa,[1] Akihiro Tamori,[1] Hiroki Sakaguchi,[1] Ayumi Sawada,[2] Setsuko Takeda,[2] Daiki Habu,[3] Susumu Shiomi[4] and Norifumi Kawada[1]

[1]*Department of Hepatology, Osaka City University Graduate School of Medicine,* [2]*Central Clinical Laboratory, Osaka City University Medical School Hospital,* [3]*Department of Medical Nutrition, Osaka City University Graduate School of Life Science, and* [4]*Department of Nuclear Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan*

Aim: The usefulness of transient elastography remains to be validated in chronic hepatitis B, particularly as a tool for monitoring the degree of liver fibrosis during treatment.

Methods: The subjects were 50 patients with chronic hepatitis B virus infection. Liver biopsy was performed in 38 patients, and in 12 patients with platelet counts of 50×10^9/L or less, cirrhosis was clinically diagnosed on the basis of specific signs of portal hypertension. Liver stiffness was measured by transient elastography at baseline and after 12 months of treatment in 20 nucleos(t)ide-naïve patients who started entecavir within 3 months after study entry.

Results: Twenty (40%) patients were classified as F1, 10 (20%) as F2, 5 (10%) as F3, and 15 (30%) as F4 (cirrhosis). Median liver stiffness (interquartile range) was 7.0 kPa (5.6–9.4), 9.8 kPa (5.6–14.7), 9.8 kPa (7.6–12.9), and 17.3 kPa (8.2–27.6) in fibrosis stages F1 to F4, respectively. Liver stiffness significantly correlated with fibrosis stage ($r = 0.46$; $P = 0.0014$). Of the patients who started entecavir, median liver stiffness significantly decreased from 11.2 kPa (7.0–15.2) to 7.8 kPa (5.1–11.9; $P = 0.0090$) during 12 months of treatment. Median levels of amino-terminal peptide of type III procollagen and type IV collagen 7S domain in serum significantly decreased from 0.9 (0.6–1.3) to 0.6 (0.5–0.7) U/mL ($P = 0.0010$) and from 5.0 (4.4–6.7) to 3.9 (3.2–4.4) ng/mL ($P = 0.015$), respectively.

Conclusion: Liver stiffness measurement can be useful for monitoring regression of liver fibrosis during entecavir treatment in patients with chronic hepatitis B virus infection.

Key words: chronic hepatitis B virus, entecavir, FibroScan, liver stiffness, transient elastography.

INTRODUCTION

INFECTION WITH HEPATITIS B virus (HBV) remains an important public health problem and a leading cause of liver-related morbidity worldwide.[1] Currently available antiviral therapy for chronic HBV includes the immunomodulator interferon and oral nucleos(t)ide analogues.[2,3] Entecavir, a cyclopentyl guanosine analogue, is the most potent agent against HBV among licensed nucleos(t)ide analogues and is used as the first-line treatment of choice for chronic HBV infection. Randomized controlled trials have demonstrated not only virological and biochemical responses, but also a histological response (defined as 2-point reduction in the Knodell necroinflammatory score without progression to fibrosis) in patients treated with entecavir.[4,5] A *post hoc* descriptive analysis of the trials showed that the stage of fibrosis was improved in 58% of nucleos(t)ide-naïve patients with advanced liver fibrosis by 52 weeks of entecavir treatment.[6]

Liver biopsy is considered the gold standard for diagnosing chronic liver disease, grading necroinflammatory activity, and staging liver fibrosis. However, sampling error can lead to underestimation of the degree of liver fibrosis, especially when biopsy specimens are small or fragmented. In addition, interpretation of the results is subject to significant intra- and inter-observer

Correspondence: Professor Norifumi Kawada, Department of Hepatology, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan. Email: kawadanori@med.osaka-cu.ac.jp

Received 18 January 2010; revision 27 April 2010; accepted 3 May 2010.

variability. Moreover, liver biopsy is not suitable for repeated evaluations, because it is invasive and can cause major complications (0.3–0.5%), including death (0.03–0.1%).[7,8] Several surrogate serum markers and laboratory indices/scores thus have been proposed as alternative techniques for the non-invasive assessment of liver fibrosis.[9,10] More recently, transient elastography (FibroScan; Echosens, Paris) has been introduced and found to be a rapid, objective, and promising technique for staging liver fibrosis by measuring liver stiffness.[11,12] In a meta-analysis of 50 studies done mainly in chronic hepatitis C, the mean areas under the receiver operating characteristic curve for the diagnosis of significant fibrosis, severe fibrosis, and cirrhosis were 0.84, 0.89, and 0.94, respectively.[13]

Compared to hepatitis C virus, the usefulness of transient elastography has been less extensively studied and validated in chronic HBV. In particular, transient elastography has not been evaluated as a tool for monitoring regression of liver fibrosis during antiviral treatment for chronic HBV, although patients' acceptance of repeated evaluations is excellent.

The aim of this study was to determine whether liver stiffness as measured by transient elastography can be used for on-treatment monitoring of the effects of entecavir on liver fibrosis in patients with chronic HBV. First, we evaluated the correlation between liver stiffness and the stage of fibrosis in 50 patients with chronic HBV. Second, in 20 patients who started entecavir within 3 months after study entry, liver stiffness, as well as serum levels of liver fibrosis markers, was measured at baseline and after 12 months of treatment.

METHODS

Patients

THE FLOW OF the participants through the trial is shown in Figure 1. Between April 2005 and June 2008, 38 patients with chronic HBV in whom liver biopsy was clinically indicated were admitted to our hospital. During the same period, 12 other patients with chronic HBV in whom percutaneous liver biopsy was contraindicated by a low platelet count ($\leq 50 \times 10^9$/L) received a clinical diagnosis of cirrhosis on the basis of specific signs of portal hypertension, such as esophageal varices.

A total of 50 consecutive patients with chronic HBV were included in this study. We excluded patients who had antibodies to hepatitis C virus and other likely causes of chronic liver diseases; ascites and other clinical signs of decompensated liver disease; acute exacerbations of liver disease, defined as a rise in alanine aminotransferase (ALT) to 10-times higher than the upper limit of normal; or hepatocellular carcinoma detectable by ultrasonography in the target area for liver stiffness measurement. Informed consent was obtained from each patient. The procedures of the study were in accordance with the Helsinki Declaration of 1975 (2000 revision).

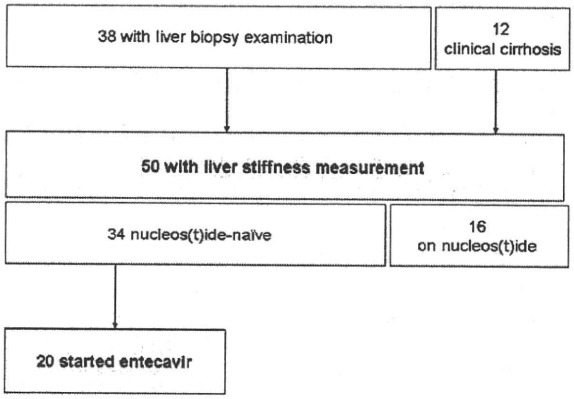

Figure 1 Flow of participants through the trial. Of the 50 patients studied, 16 were receiving nucleos(t)ide analogue treatment at entry. Of the remaining 34 nucleos(t)ide-naïve patients, 20 patients started entecavir within 3 months of entry.

Liver histology and quantification of liver fibrosis

Liver biopsies were performed with a 15-gauge Tru-Cut needle (Hakko, Tokyo) under ultrasound guidance. Liver tissues were fixed in formalin immediately after biopsy and embedded in paraffin. Four-micrometre-thick sections were stained with hematoxylin-eosin and Sirius red and immunostained with α-smooth muscle actin (Dako, Glostrup). The stage of liver fibrosis and grade of necroinflammatory activity were evaluated semiquantitatively according to the METAVIR scoring system as follows: F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.[14] METAVIR activity scores were defined as follows: A1, mild; A2, moderate; and A3, severe. A liver sample was considered adequate if it was longer than 15 mm and contained six or more portal tracts.

In some biopsy specimens, morphometric image analysis was performed with a computerized system, consisting of a photomicroscope, digital camera, and

LuminaVision 2.4 bio-imaging software (Mitani, Tokyo), to quantitatively assess fibrosis. The proportion of area stained with Sirius red or α-smooth muscle actin in liver-biopsy sections was calculated as the sum of the pixel-wise bound stain measurements divided by the number of summed pixels.

Liver stiffness measurement

Liver stiffness was measured by transient elastography (FibroScan; EchoSens)[11,12] within 3 months of study entry. Briefly, this system is equipped with a probe including an ultrasonic transducer mounted on the axis of a vibrator. The subject laid down on a bed in the horizontal supine position with the right arm in maximal abduction, and a probe was placed on the skin above the right intercostal space. A vibration transmitted from the vibrator toward the tissue induces an elastic shear wave that propagates through the right lobe of the liver. These propagations are followed by pulse-echo ultrasound acquisitions, and their velocity, which is directly related to tissue stiffness, is measured. The median of ten successful measurements was expressed in units of kilopascals (kPa) and used as the liver stiffness for a given subject. Performance was considered optimal when the rate of successful measurements to the total number of acquisitions was at least 60% and the ratio of the interquartile range to the median value did not exceed 30%.

Biochemical, hematological, and virological examinations

The following variables were determined at baseline: serum ALT activity, platelet count, HBV surface antigen, HBV e antigen (HBeAg), anti-HBe, HBV genotypes, and HBV DNA levels. HBV surface antigen, HBeAg, and anti-HBe were detected by chemiluminescence enzyme immunoassay. Genotypes of HBV were identified by enzyme-linked immunosorbent assay (Institute of Immunology, Tokyo).[15] HBV DNA was measured by transcription-mediated amplification with a hybridization protection assay (Chugai Diagnostics, Tokyo); the detection range was 3.7–8.7 $\log_{10}$ copies/mL.[16] If HBV DNA was not detected by this method, a PCR-based Amplicor Monitor test (Roche Molecular Systems, Pleasanton, CA) was utilized; the detection range was 2.6–7.6 $\log_{10}$ copies/mL.[17]

Surrogate serum markers of liver fibrosis

In patients who started entecavir within 3 months of entry, two serum markers of liver fibrosis were measured at baseline and after 12 months of treatment. Serum levels of amino-terminal peptide of type III procollagen (PIIINP) were measured by radioimmunoassay (Nihon Schering K.K., Osaka), with a normal range of 0.3–0.8 U/mL. Serum levels of type IV collagen 7S were measured by radioimmunoassay (Mitsubishi Kagaku Iatron, Tokyo), with a normal range of not more than 6 ng/mL.

Statistical analysis

Statistical analysis was performed with the Statview SE + Graphics program, version 5.0 (SAS Institute, Cary, NC). Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Differences in proportions were evaluated by Fisher's exact test. The significance of correlation was tested by Spearman's rank analysis. The significance of changes in values between two time points was evaluated by the Wilcoxon signed-rank test. A two-tailed *P*-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Baseline characteristics of patients

TABLE 1 SHOWS the baseline characteristics of the enrolled patients with chronic HBV. Mean body mass index was 22.4 kg/m^2; six patients were overweight (25–30 kg/m^2), and no patient was obese (>30 kg/m^2). Among all 50 subjects, 16 were receiving nucleos(t)ide analogue treatment at entry: 5 were receiving lamivudine, 2 lamivudine plus adefovir dipivoxil, and 9 entecavir (Fig. 1). Among the remaining 34 nucleos(t)ide-naïve patients, 20 started entecavir at an oral dose of 0.5 mg once daily within 3 months after entry. The 20 patients who started entecavir had significantly higher ALT ($P = 0.018$) and HBV DNA levels ($P = 0.011$) and a lower stage of fibrosis ($P = 0.0016$) than the other 30 patients.

Liver stiffness and fibrosis stage

The liver stiffness measurements are shown according to the stage of fibrosis in Figure 2. Of the 50 enrolled patients with chronic HBV, 20 (40%) were classified as F1, 10 (20%) as F2, 5 (10%) as F3, and 3 (6%) as F4 on liver biopsies; cirrhosis was clinically diagnosed in 12 (24%) patients. When clinical cirrhosis was combined with histological F4, median liver stiffness (interquartile range) was 7.0 kPa (5.6–9.4), 9.8 kPa (5.6–14.7), 9.8 kPa (7.6–12.9), and 17.3 kPa (8.2–27.6) in fibrosis stages F1 to F4, respectively. Liver stiffness significantly correlated with the stage of fibrosis ($r = 0.46$;

Table 1 Baseline characteristics of patients

	All patients ($n = 50$)	Patients who started entecavir ($n = 20$)
Age (years)†	50 ± 13	50 ± 14
Sex (male/female)‡	28/22	14/6
Body mass index (kg/m^2)†	22.4 ± 2.7	22.1 ± 2.8
ALT (IU/L)§	75 (40–125)	111 (67–186)
Platelet count (×10^9/L)†	158 ± 63	152 ± 57
HBeAg (+/−)‡	27/23	8/12
HBV DNA ($\log_{10}$ copies/mL)†	5.9 ± 2.1	7.0 ± 1.1
HBV genotype (A/B/C/D)‡	1/4/39/0	1/2/17/0
Grade of necroinflammation‡ (A1/A2/A3)	19/17/2	10/6/1
Stage of fibrosis‡ (F1/F2/F3/F4¶)	20/10/5/15	6/8/4/2

†Mean ± SD; ‡Numbers of patients; §Median (interquartile range). ¶F4 includes cirrhosis clinically diagnosed on the basis of specific signs.
ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

$P = 0.0014$). At the F1 to F4 stages of fibrosis, there was no significant correlation of liver stiffness with serum ALT levels ($r = 0.40$, 0.097, 0.60, and 0.11; $P = 0.083$, 0.77, 0.23, and 0.69, respectively) or with histological necroinflammatory activity ($r = 0.10$, 0.12, 0.71, and 0.50; $P = 0.67$, 0.73, 0.16, and 0.48, respectively).

Liver stiffness during entecavir treatment

Among the 20 patients who started entecavir, 6 (30%) were histologically classified as F1, 8 (40%) as F2, 4 (20%) as F3, and 1 (5%) as F4; cirrhosis was clinically diagnosed in 1 (5%) patient. Serum HBV DNA levels decreased to below the lower detection limit of the PCR assay in 8 (40%) patients at 3 months, 16 (80%) patients at 6 months, and 19 (95%) patients at 12 months. Serum ALT levels decreased to within the reference range in 5 (25%) patients at 3 months, 12 (60%) patients at 6 months, and 14 (70%) patients at 12 months. The changes in liver stiffness during the first 12 months of entecavir treatment are shown in Figure 3. Median liver stiffness (interquartile range) significantly decreased from 11.2 (7.0–15.2) kPa to 7.8 (5.1–11.9) kPa ($P = 0.0090$) during the 12 months. The rate of decrease in liver stiffness did not correlate with the rate of decrease in serum ALT levels ($r = 0.38$; $P = 0.88$).

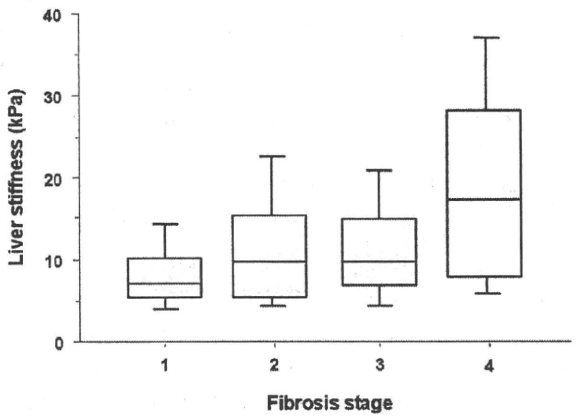

Figure 2 Box plots of liver stiffness measurements according to the stage of fibrosis in all patients with chronic hepatitis B virus. The length of the box represents the interquartile range, within which 50% of the values are located. The lines in the boxes represent the median values. The error bars represent the minimum and maximum values (range). The stage of fibrosis significantly correlated with liver stiffness ($r = 0.46$; $P = 0.0014$).

Serum fibrosis markers during entecavir treatment

The changes in serum fibrosis marker levels during the first 12 months of therapy in the 20 patients treated with entecavir are shown in Figure 4. Median PIIINP levels significantly decreased from 0.9 (0.6–1.3) U/mL to 0.6 (0.5–0.7) U/mL ($P = 0.0010$). Median levels of type IV collagen 7S domain significantly decreased from 5.0 ng/mL (4.4–6.7) to 3.9 ng/mL (3.2–4.4; $P = 0.015$). The rate of decrease in liver stiffness significantly correlated with the rate of decrease in serum PIIINP levels ($r = 0.46$; $P = 0.040$), but not with the rate of decrease in type IV collagen 7S domain levels ($r = 0.27$; $P = 0.26$).

Case presentations

Figures 5 and 6 show the results of paired liver biopsies in two patients, performed at baseline and after

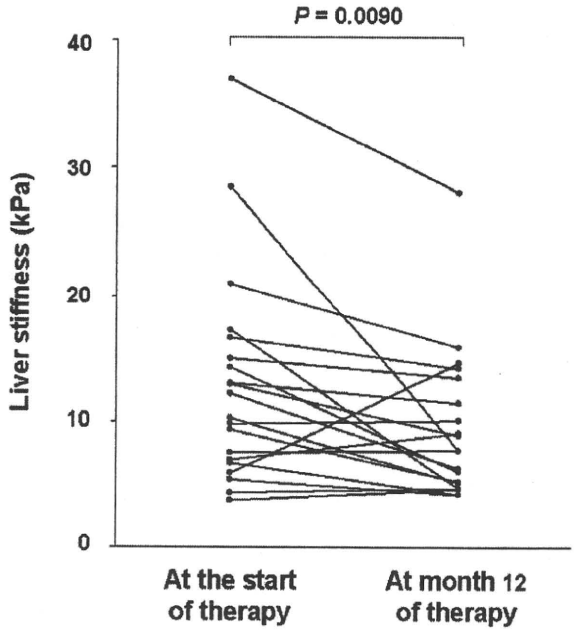

Figure 3 Changes in liver stiffness during the first 12 months of therapy in the 20 patients treated with entecavir. Median liver stiffness (interquartile range) significantly decreased from 11.2 kPa (7.0–15.2) to 7.8 kPa (5.1–11.9; $P = 0.0090$) during the 12 months.

12 months of entecavir treatment. One patient (case 1) was a 41-year-old man in whom the percentage decrease in liver stiffness was highest during the 12 months of treatment (28.4 kPa at baseline, 7.8 kPa at 12 months). The pretreatment HBV DNA level was 7.4 $\log_{10}$ copies/mL and became undetectable on PCR by month 6. The ALT activity was 215 IU/L and fell to the normal range by month 6. The histological grade and stage improved from A2/F4 to A1/F3. The proportion of fibrosis area on morphometric analysis decreased from 12.3% to 8.3% in specimens stained with Sirius red (Fig. 5a) and from 7.0% to 3.7% in specimens stained with α-smooth muscle actin (Fig. 5b). Serum markers of liver fibrosis also decreased (PIIINP, 1.4 U/mL to 0.5 U/mL; type IV collagen 7S domain, 7.1 ng/mL to 3.8 ng/mL).

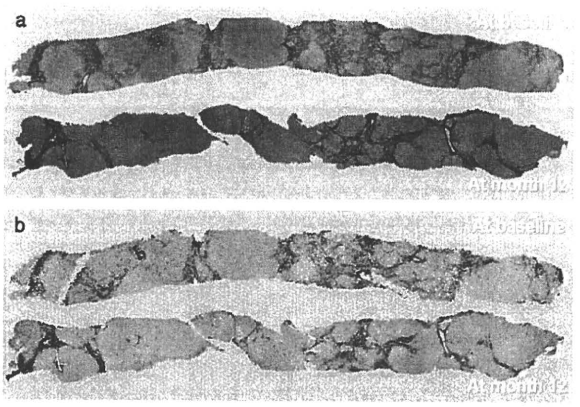

Figure 5 Paired liver biopsies performed in case 1 at baseline and after 12 months of entecavir treatment. The proportion of fibrosis area on morphometry decreased from (a) 12.3% at baseline to 8.3% after 12 months of therapy in specimens stained with Sirius red, and (b) from 7.0% to 3.7% in specimens stained with α-smooth muscle actin.

The other patient (case 2) was a 45-year-old man who had the greatest increase in liver stiffness (6.0 kPa at

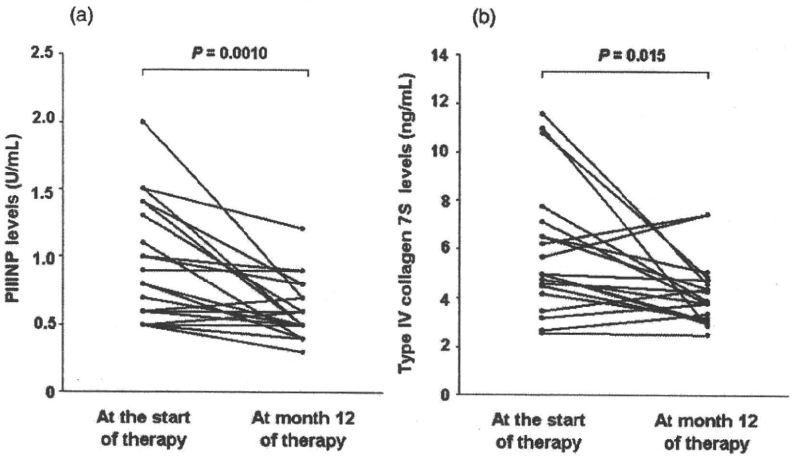

Figure 4 Changes in serum fibrosis marker levels during the first 12 months of therapy in the 20 patients treated with entecavir. There were significant decreases in the levels of (a) peptide of type III procollagen (PIIINP; $P = 0.0010$) and (b) type IV collagen 7S domain in serum ($P = 0.015$).

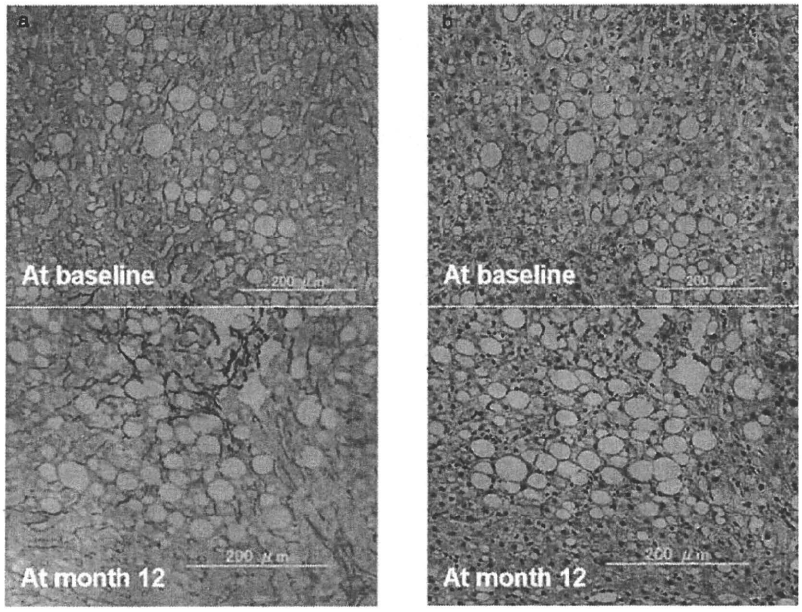

Figure 6 Paired liver biopsies performed in case 2 at baseline and after 12 months of entecavir treatment. The proportion of fibrosis area on morphometry increased from (a) 3.4% to 4.4% on Sirius red staining, and (b) from 0.9% to 1.5% on α-smooth muscle actin staining. At baseline, hepatic steatosis involved less than 5% of the biopsy specimen; the presence of steatosis was noted at the second biopsy involving about 20% of the specimen. The perisinusoidal/pericellular fibrosis was seen around hepatocytes distended by steatosis.

baseline to 14.5 kPa at 12 months). He did not drink alcohol, but body weight increased by 3.0 kg during the 12 months of treatment. Although the HBV DNA level rapidly decreased from 7.6 $\log_{10}$ copies/mL to below the detection limit by month 7, ALT activity did not fall significantly from baseline value (41 IU/L). The results of histological evaluations were unchanged on the paired biopsies performed 12 months apart (A1/F2). The proportion of fibrosis area on morphometric analysis increased from 3.4% to 4.4% on Sirius red staining (Fig. 6a) and from 0.9% to 1.5% on α-smooth muscle actin staining (Fig. 6b). On the second biopsy, the presence of hepatic steatosis was noted, involving about 20% of the biopsy specimen; perisinusoidal/pericellular fibrosis was seen around hepatocytes distended by steatosis. Serum fibrosis markers also did not decrease (PIIINP, 0.9 U/mL to 0.9 U/mL; type IV collagen 7S domain, 4.8 ng/mL to 5.0 ng/mL).

DISCUSSION

OF THE 50 patients studied, 45 were Japanese, two were Chinese, two Korean, and one Philippine. In Japan and other countries in east Asia, genotype C is the most prevalent type of HBV,[18,19] and most patients with chronic HBV acquire the virus perinatally or in early childhood.[20] The rates of virological and biochemical responses to interferon are thus lower than those reported in Europe and the United States. Guidelines proposed by the Japanese Study Group of the Standardization of Treatment of Viral Hepatitis Including Cirrhosis recommend that nucleos(t)ide-naïve patients with chronic HBV who are 35 years or older should receive entecavir as the treatment of choice.[21] In our patients, characterized by a predominance of genotype C, the rate of response to entecavir was similar to the rates obtained in randomized controlled trials conducted worldwide.[4,5]

Liver stiffness as measured by transient elastography significantly correlated with the stage of fibrosis in our patients with chronic HBV (Fig. 2), consistent with the results of previous studies.[12,13] Factors other than fibrosis, including necroinflammatory activity,[22–24] obesity,[25] and extrahepatic cholestasis,[26] can affect the results of liver stiffness measurement. In particular, chronic HBV is associated with more frequent acute exacerbations than chronic hepatitis C. In this study, we did not include patients with acute exacerbation. There was no significant correlation between liver stiffness and serum ALT levels or histological necroinflammatory activity. No patient was obese as defined by a body mass index of >30 kg/m^2.

Liver stiffness measurement is generally less accurate for the diagnosis of liver fibrosis in chronic HBV than in chronic hepatitis C.[27] In previous studies that included both chronic HBV and C,[28,29] median liver stiffness at each stage of fibrosis was lower in chronic HBV than in

chronic hepatitis C. The reported cutoff value for predicting cirrhosis in chronic HBV ranged from 9.0 to 11.0 kPa,[30,31] which is lower than 13.0 kPa, the optimal cutoff value based on a meta-analysis of 17 studies of various chronic liver diseases (mostly chronic hepatitis C).[13] In the present study, liver stiffness was lower than 13.0 kPa in 5 (33%) of the 15 patients with cirrhosis (data not shown). In addition, it was difficult to predict advanced fibrosis (≥F3), since median liver stiffness was similar in F2 and F3 (9.8 kPa). One possible explanation for difference in diagnostic accuracy is that the amount of fibrosis in the cirrhotic liver is lower in chronic HBV than in chronic hepatitis C because macronodular cirrhosis, characterized by large nodules delimited by thin septa, is more common in patients chronically infected with HBV.

Median liver stiffness as measured by transient elastography significantly decreased during the 12 months of entecavir treatment (Fig. 3). Our results may reflect an improvement in liver fibrosis by treatment with entecavir, similar to that demonstrated in a *post hoc* descriptive analysis of randomized controlled trials.[6] To exclude the possibility that the decrease in liver stiffness was caused by regression of necroinflammatory activity, we also measured the levels of serum markers of liver fibrosis. In general, liver fibrosis markers can be divided into two groups, either direct or indirect. Direct markers of fibrosis reflect serum extracellular matrix turnover. For example, PIIINP, a product formed by cleavage of procollagen III, is released into the serum during matrix deposition.[32] Type IV collagen 7S domain is located in basement membranes and released during interstitial filament degradation, thereby reflecting matrix degradation.[33] In this study, both markers significantly decreased during the 12 months of entecavir treatment (Fig. 4).

Indirect markers of liver fibrosis reflect alterations in hepatic function, but do not directly reflect hepatic extracellular matrix metabolism. Indirect markers include platelet count, the results of coagulation studies, hepatic aminotransferases, and combined indices/scores derived from these variables. Combined indices/scores such as aspartate aminotransferase/ALT ratio,[34] aspartate aminotransferase-to-platelet ratio index,[35] cirrhosis discriminant score,[36] and Lok index,[37] are not suitable for on-treatment assessment of liver fibrosis in chronic HBV, because these indices/scores include hepatic aminotransferases, which rapidly decrease after the start of antiviral treatment.

Many studies have shown significant correlations between the results of morphometric image analysis and those of semiquantitative histological staging,[38,39] although potential limitations of morphometry include the sampling error. On liver biopsy, only 1/50 000 of the organ (and 1/100 of the region of interest for liver stiffness measurement) is analyzed. In addition, to accurately measure liver fibrosis, it is necessary to carefully exclude necroinflammation in fibrous areas, requiring significant time and labor, even for a trained hepatopathologist. We did a morphometric analysis in two patients with paired liver biopsy specimens stained with Sirius red for collagen or with α-smooth muscle actin for activated hepatic stellate cells. One patient (case 1) showed regression of both histological stage and extent of fibrosis on morphometry. The results of liver stiffness measurement and serum fibrosis markers also improved. More interestingly, in the other patient (case 2), both the extent of fibrosis as estimated by morphometry and liver stiffness increased, despite a virological response to entecavir. We speculate that pericellular fibrosis caused by steatohepatitis was the main cause of increase in liver stiffness in this patient. In the previous study,[40] liver stiffness correlated more strongly with pericellular fibrosis than with periportal or perivenular fibrosis. The histological stage of fibrosis was unchanged, probably because it reflects liver architectural abnormalities, not directly the amount of fibrosis. If liver stiffness increases during antivairal treatment for chronic HBV, liver biopsy should be considered to exclude other potential causes of chronic liver disease.

The major limitation of this study is the short duration of observation during entecavir treatment. In the 5 patients treated with entecavir for more than 24 months, median liver stiffness decreased slightly, but not significantly from 7.8 kPa to 7.0 kPa during the second 12 months of entecavir treatment (data not shown). The decrease in liver stiffness during the second 12 months of treatment might be attributed solely to an improvement in liver fibrosis, not an improvement in necroinflammation. Another limitation of this study is the small number of patients. The rate of decrease in liver stiffness significantly correlated with rate of decrease in the serum level of PIIINP, but not with that of type IV collagen 7S domain, possibly because of an insufficient number of patients. Larger studies are required to confirm the usefulness of transient elastography as a tool for on-treatment monitoring of the regression of liver fibrosis.

In conclusion, transient elastography is a rapid, noninvasive, objective, and promising technique for the assessment of fibrosis by measuring liver stiffness in

patients with chronic HBV, as well as those with chronic hepatitis C virus. Liver stiffness measurement might be useful for monitoring regression of liver fibrosis during entecavir treatment for chronic HBV.

ACKNOWLEDGMENTS

THE AUTHORS ARE grateful to Ms Sanae Deguchi and Ms Rie Yasuda for technical assistance.

REFERENCES

1 Dienstag JL. Hepatitis B virus infection. *N Engl J Med* 2008; **359**: 1486–500.
2 Lok AS, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; **50**: 661–2.
3 European Association for The Study of the Liver. EASL Clinical Practice Guidelines: management of chronic hepatitis B. *J Hepatol* 2009; **50**: 227–42.
4 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001–10.
5 Lai CL, Shouval D, Lok AS *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; **354**: 1011–20.
6 Schiff E, Simsek H, Lee WM *et al.* Efficacy and safety of entecavir in patients with chronic hepatitis B and advanced hepatic fibrosis or cirrhosis. *Am J Gastroenterol* 2008; **103**: 2776–83.
7 Perrault J, McGill DB, Ott BJ, Taylor WF. Liver biopsy: complications in 1000 inpatients and outpatients. *Gastroenterology* 1978; **74**: 103–6.
8 Janes CH, Lindor KD. Outcome of patients hospitalized for complications after outpatient liver biopsy. *Ann Intern Med* 1993; **118**: 96–8.
9 Afdhal NH, Nunes D. Evaluation of liver fibrosis: a concise review. *Am J Gastroenterol* 2004; **99**: 1160–74.
10 Pinzani M, Vizzutti F, Arena U, Marra F. Technology Insight: noninvasive assessment of liver fibrosis by biochemical scores and elastography. *Nat Clin Pract Gastroenterol Hepatol* 2008; **5**: 95–106.
11 Yoshioka K, Kawabe N, Hashimoto S. Transient elastography: applications and limitations. *Hepatol Res* 2008; **38**: 1063–8.
12 Castera L, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. *J Hepatol* 2008; **48**: 835–47.
13 Friedrich-Rust M, Ong MF, Martens S *et al.* Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. *Gastroenterology* 2008; **134**: 960–74.
14 Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289–93.
15 Usuda S, Okamoto H, Iwanari H *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods* 1999; **80**: 97–112.
16 Ide T, Kumashiro R, Hino T *et al.* Transcription-mediated amplification is more useful in the follow-up of patients with chronic hepatitis B treated with lamivudine. *Hepatol Res* 2001; **21**: 76–84.
17 Gerken G, Gomes J, Lampertico P *et al.* Clinical evaluation and applications of the Amplicor HBV Monitor test, a quantitative HBV DNA PCR assay. *J Virol Methods* 1998; **74**: 155–65.
18 Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34**: 590–4.
19 Enomoto M, Tamori A, Nishiguchi S. Hepatitis B virus genotypes and response to antiviral therapy. *Clin Lab* 2006; **52**: 43–7.
20 Liaw YF, Leung N, Kao JH *et al.* Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol Int* 2008; **2**: 263–83.
21 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40**: 1–7.
22 Arena U, Vizzutti F, Corti G *et al.* Acute viral hepatitis increases liver stiffness values measured by transient elastography. *Hepatology* 2008; **47**: 380–4.
23 Sagir A, Erhardt A, Schmitt M, Häussinger D. Transient elastography is unreliable for detection of cirrhosis in patients with acute liver damage. *Hepatology* 2008; **47**: 592–5.
24 Wong GL, Wong VW, Choi PC *et al.* Increased liver stiffness measurement by transient elastography in severe acute exacerbation of chronic hepatitis B. *J Gastroenterol Hepatol* 2009; **24**: 1002–7.
25 Fraquelli M, Rigamonti C, Casazza G *et al.* Reproducibility of transient elastography in the evaluation of liver fibrosis in patients with chronic liver disease. *Gut* 2007; **56**: 968–73.
26 Millonig G, Reimann FM, Friedrich S *et al.* Extrahepatic cholestasis increases liver stiffness (FibroScan) irrespective of fibrosis. *Hepatology* 2008; **48**: 1718–23.
27 Ganne-Carrié N, Ziol M, de Ledinghen V *et al.* Accuracy of liver stiffness measurement for the diagnosis of cirrhosis in patients with chronic liver diseases. *Hepatology* 2006; **44**: 1511–7.
28 Coco B, Oliveri F, Maina AM *et al.* Transient elastography: a new surrogate marker of liver fibrosis influenced by major changes of transaminases. *J Viral Hepat* 2007; **14**: 360–9.
29 Ogawa E, Furusyo N, Toyoda K *et al.* Transient elastography for patients with chronic hepatitis B and C virus infec-

tion: Non-invasive, quantitative assessment of liver fibrosis. *Hepatol Res* 2007; **37**: 1002–10.
30 Chan HL, Wong GL, Choi PC *et al.* Alanine aminotransferase-based algorithms of liver stiffness measurement by transient elastography (Fibroscan) for liver fibrosis in chronic hepatitis B. *J Viral Hepat* 2009; **16**: 36–44.
31 Marcellin P, Ziol M, Bedossa P *et al.* Non-invasive assessment of liver fibrosis by stiffness measurement in patients with chronic hepatitis B. *Liver Int* 2009; **29**: 242–7.
32 McCullough AJ, Stassen WN, Wiesner RH, Czaja AJ. Serum type III procollagen peptide concentrations in severe chronic active hepatitis: relationship to cirrhosis and disease activity. *Hepatology* 1987; **7**: 49–54.
33 Murawaki Y, Ikuta Y, Koda M, Yamada S, Kawasaki H. Comparison of serum 7S fragment of type IV collagen and serum central triple-helix of type IV collagen for assessment of liver fibrosis in patients with chronic viral liver disease. *J Hepatol* 1996; **24**: 148–54.
34 Williams AL, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. *Gastroenterology* 1988; **95**: 734–9.
35 Wai CT, Greenson JK, Fontana RJ *et al.* A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 518–26.
36 Bonacini M, Hadi G, Govindarajan S, Lindsay KL. Utility of a discriminant score for diagnosing advanced fibrosis or cirrhosis in patients with chronic hepatitis C virus infection. *Am J Gastroenterol* 1997; **92**: 1302–4.
37 Lok AS, Ghany MG, Goodman ZD *et al.* Predicting cirrhosis in patients with hepatitis C based on standard laboratory tests: results of the HALT-C cohort. *Hepatology* 2005; **42**: 282–92.
38 Goodman ZD, Becker RL, Pockros PJ *et al.* Progression of fibrosis in advanced chronic hepatitis C: evaluation by morphometric image analysis. *Hepatology* 2007; **45**: 886–94.
39 Nitta Y, Kawabe N, Hashimoto S *et al.* Liver stiffness measured by transient elastography correlates with fibrosis area in liver biopsy in patients with chronic hepatitis C. *Hepatol Res* 2009; **39**: 675–84.
40 Wong GL, Wong VW, Choi PC *et al.* Assessment of fibrosis by transient elastography compared with liver biopsy and morphometry in chronic liver diseases. *Clin Gastroenterol Hepatol* 2008; **6**: 1027–35.

J Gastroenterol (2011) 46:350–358
DOI 10.1007/s00535-010-0301-x

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Real-time tissue elastography as a tool for the noninvasive assessment of liver stiffness in patients with chronic hepatitis C

Hiroyasu Morikawa · Katsuhiko Fukuda · Sawako Kobayashi · Hideki Fujii · Shuji Iwai · Masaru Enomoto · Akihiro Tamori · Hiroki Sakaguchi · Norifumi Kawada

Received: 6 May 2010 / Accepted: 15 July 2010 / Published online: 10 August 2010

Abstract
Background Although histopathological examination by "invasive" liver biopsy remains the gold standard for evaluating disease progression in chronic liver disease, noninvasive tools have appeared and have led to great progress in diagnosing the stage of hepatic fibrosis. The aim of this study was to assess the value of real-time tissue elastography, using an instrument made in Japan, for the visible measurement of liver elasticity; in particular, comparing the results with those of transient elastography (Fibroscan).
Methods Real-time tissue elastography (RTE), transient elastography (Fibroscan), liver biopsy, and routine laboratory analyses were performed in 101 patients with chronic hepatitis C. The values for tissue elasticity obtained using novel software (Elasto_ver 1.5.1) connected to RTE were transferred to four image features, Mean, Standard Deviation (SD), Area, and Complexity. Their association with the stage of fibrosis at biopsy and with liver stiffness (kPa) obtained by Fibroscan was analyzed.
Results Colored images obtained by RTE were classified into diffuse soft, intermediate, and patchy hard patterns and the calculated elasticity differed significantly between patients according to and correlated with the stages of fibrosis ($p < 0.0001$). Mean, SD, Area, and Complexity showed significant differences between the stages of fibrosis (Tukey–Kramer test, $p < 0.05$). In discriminating patients with cirrhosis, the areas under the receiver operating characteristic curves (AUC) were 0.91 for Mean, 0.84 for SD, 0.91 for Area, 0.93 for Complexity, and 0.95 for Fibroscan.
Conclusions RTE is a noninvasive instrument for the colored visualization of liver elasticity in patients with chronic liver disease.

Keywords Liver fibrosis · Transient elastography · Ultrasound · Liver biopsy

H. Morikawa · S. Kobayashi · H. Fujii · S. Iwai · M. Enomoto · A. Tamori · H. Sakaguchi · N. Kawada (✉)
Department of Hepatology, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno, Osaka 545-8585, Japan
e-mail: kawadanori@med.osaka-cu.ac.jp

K. Fukuda
Department of Gastroenterology, PL Hospital, Tondabayashi, Japan

Introduction

Hepatitis C virus (HCV) infects approximately 170 million individuals worldwide, according to a report from the World Health Organization [1]. Chronic liver damage attributable to HCV infection results in hepatic fibrosis, which is characterized by an unusual accumulation of extracellular matrix materials produced by fibroblast-like cells including stellate cells in the hepatic parenchyma. Hepatic fibrosis progresses towards cirrhosis, an end-stage liver injury, leading to hepatic failure, hepatocellular carcinoma, and finally death. Thus, precise evaluation of the stage of chronic hepatitis C with respect to fibrosis has become an important issue to prevent the occurrence of cirrhosis and to initiate appropriate therapeutic intervention such as viral eradication using pegylated interferon (PEG-IFN) plus ribavirin [2].

Although liver biopsy is acknowledged as the gold standard for staging disease progression, there are some limitations, including its invasiveness, risk of complications, sampling error, variability in histopathological interpretation, and the reluctance of patients to submit to repeated examinations [3]. Because of these disadvantages, there is a

growing shift in clinical practice to utilize or develop 'non-invasive' methodologies to reflect the stage of liver fibrosis.

Several noninvasive approaches have appeared, such as serum fibrosis markers, transient elastography (Fibroscan®; Echosens SA, Paris, France), and real-time tissue elastography (RTE). Serum fibrosis markers include direct tests, such as hyaluronic acid and type IV collagen, and indirect approaches, which detect alterations in hepatic function but do not directly reflect hepatic extracellular matrix metabolism; these include platelet counts, coagulation studies, and hepatic transaminases, or their combinations in indices/scores, such as the aspartate aminotransferase-to-platelet ratio index (APRI) [4, 5].

Transient elastography, which has been developed for the measurement of liver stiffness, is considered to reflect more directly than other means the fibrotic evolution of chronic liver trauma [6–10]. In 2005, Castera et al. and Ziol et al. reported that liver stiffness measurements could be useful in assessing the presence of significant fibrosis (F2–4) and in suggesting the presence of cirrhosis in cohorts of patients with chronic hepatitis C; the areas under the receiver operating characteristic curves (AUCs) ranged from 0.79 to 0.83 for the prediction of F2–4 and were over 0.95 for the identification of cirrhosis [11, 12]. Friedrich-Rust et al. [13] assessed the overall performance of transient elastography for the diagnosis of liver fibrosis by a meta-analysis which included fifty articles; the mean AUCs for the diagnosis of significant fibrosis, severe fibrosis, and cirrhosis were 0.84, 0.89, and 0.94, respectively. The limitations of this method have also been discussed; intraobserver agreement is influenced by variables such as body mass index (BMI, particularly when ≥28), hepatic steatosis, and flares of transaminases [11–14].

RTE is a method developed in Japan for the visual assessment of tissue elasticity integrated in a sonography machine, based on a Combined Autocorrelation Method that calculates the relative hardness of tissue rapidly from the degree of tissue distortion and which displays this information as a color image. The distortion of tissue is color-coded according to its magnitude and superimposed translucently on a conventional B-mode image. This simultaneous display enables us to evaluate the anatomical correspondence between tissue elasticity and B-mode images. The RTE image is constructed by the amount of displacement of the reflected ultrasound echoes under compression. Ultrasound elastography does not demonstrate physical elasticity directly, but shows the relative degree of tissue strain when subtle compression is applied. In hard tissue, the amount of displacement of the reflected ultrasound echoes is low, whereas in soft tissue, the amount of displacement is higher because soft tissue can be compressed more than hard tissue [15, 16]. This technology has already been proved to be diagnostically valuable in detecting space-occupying lesions in the breast, prostate, and pancreas [17–20]. Friedrich-Rust et al. [21] attempted to determine the elasticity of liver tissue in 79 patients with chronic viral hepatitis. They developed an elasticity score from the color-coded bit-map image produced by the computer program Mathlab version 6 (Math Works, Natick, MA, US). However, the diagnostic accuracy of this semi-quantitative assessment for the prediction of significant fibrosis (METAVIR scoring system ≥F2), severe fibrosis (≥F3), and cirrhosis (F4) was not satisfactory; the AUCs were 0.75, 0.73, and 0.69, respectively [21].

We report here an updated RTE system as a tool for the noninvasive assessment of liver stiffness in patients with chronic hepatitis C. In this new system, all pixel data in the colored image were transformed into a histogram and a binary image for more accurate quantification, using an exclusive software program.

Methods

Patients

Ten healthy adult volunteers with no evident liver disease were recruited after giving their oral informed consent. One hundred and one patients with chronic hepatitis C, whose disease was defined by the presence of serum anti-hepatitis C virus (HCV) antibodies and serum HCV RNA, with serum levels of alanine aminotransferase above the upper limit of normal, were included in this study. Percutaneous liver biopsy or laparoscopy was performed on the patients within 1 week following Fibroscan and RTE analysis at the Department of Hepatology, Osaka City University Hospital, between September 2007 and September 2009. The study protocol accorded with the Helsinki Declaration and was approved by the ethics committee of our institution. Patients were enrolled and liver biopsy was performed after informed consent was obtained.

Liver histology and quantification of liver fibrosis

Liver biopsy was carried out using a 15-gauge Tru-Cut needle biopsy apparatus (Hakko, Tokyo, Japan). The median length of liver samples obtained at biopsy was 18 mm (range 10–25 mm). Tissue specimens obtained by liver biopsy were fixed immediately in 10% formalin solution and embedded in paraffin. After cutting, sections were stained with hematoxylin and eosin or Azan Mallory and the stage of fibrosis and grade of inflammatory activity in the liver were determined according to the METAVIR scoring system [22, 23]. All biopsy specimens were examined independently by two experienced pathologists who were blinded to the clinical data and the measurements