**Table 2** Comparison of ART and CAM for the detection of HCV RNA in the serum of 13 patients with virological breakthrough by ART

| Patients | ART (week) | | | | | | CAM (week) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A, H[a], HCV RNA | 4 | 12 | 24 | 36 | 48 | EOF | 4 | 12 | 24 | 36 | 48 | EOF |
| 1. 55M, F1, 5.1 log | + | − | − | + | − | + | + | + | − | − | − | + |
| 2. 60M, NT, 5.9 log | + | − | − | − | + | + | + | + | − | − | − | + |
| 3. 55F, F1, 5.5 log | + | − | − | + | − | + | + | + | − | − | − | + |
| 4. 71F, F2, 5.6 log | + | − | − | + | − | + | + | + | − | − | − | + |
| 5. 64F, F1, 5.0 log | + | − | − | + | − | + | + | − | − | − | − | + |
| 6. 54F, F1, 5.8 log | + | − | + | − | − | + | − | − | − | − | − | + |
| 7. 56F, F2, 5.1 log | + | + | − | − | + | + | − | − | − | − | − | + |
| 8. 66F, F0, 5.0 log | + | + | − | + | − | + | + | − | − | − | − | + |
| 9. 65M, F3, 6.1 log | + | + | − | − | + | + | + | + | − | − | − | + |
| 10. 35F, NT, 6.4 log | + | + | − | + | − | + | + | + | − | − | − | + |
| 11. 58F, F3, 5.1 log | + | + | − | − | + | + | − | − | − | − | − | + |
| 12. 67F, F2, 6.2 log | + | + | − | + | + | + | + | + | − | − | − | + |
| 13. 68F, F2, 6.4 log | + | + | + | − | + | + | + | + | − | − | − | + |

*HCV* hepatitis C virus, *ART* Abbot RealTime PCR assay, *CAM* Roche COBAS Amplicor Monitor, *A* age (years) and sex (M/F), *H* histology, *NT* not tested, *EOF* end of follow-up (week 24 after end of treatment)

[a] Fibrosis only

**Table 3** Comparison of ART and CAM for the detection of HCV RNA in the serum of 3 patients with virological breakthrough by CAM

| Patients | CAM (week) | | | | | | ART (week) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A, H[a], HCV RNA | 4 | 12 | 24 | 36 | 48 | EOF | 4 | 12 | 24 | 36 | 48 | EOF |
| 1. 55M, F1, 5.1 log | + | − | + | − | − | + | + | + | + | − | − | + |
| 2. 64F, F1, 5.9 log | + | − | + | − | − | + | + | + | + | + | + | + |
| 3. 58M, F1, 6.0 log | + | + | − | + | − | + | + | + | + | + | + | + |

*HCV* hepatitis C virus, *ART* Abbot RealTime PCR assay, *CAM* Roche COBAS Amplicor Monitor, *A* age (years) and sex (M/F), *H* histology, *EOF* end of follow-up (week 24 after end of treatment)

[a] Fibrosis only

determined by ART were lower than those by CAM at all testing time points (ART, 70.4, 86.5, 98.6, 100, and 100%, vs. CAM, 80.5, 98.4, 100, 100, and 100%, respectively), but there were no significant differences.

### Comparison of HCV RNA detection by ART and CAM among patients with virological breakthrough

We defined virological breakthrough as undetectable HCV RNA during the treatment and the reappearance of serum HCV RNA positivity. Both ART and CAM analyses were done of the HCV viremia of patients who had a virological breakthrough. Virological breakthrough was detected by ART in 13 patients and by CAM in 3 patients. Table 2 shows the transition of HCV RNA detection for the 13 patients with virological breakthrough detected by ART, and Table 3 shows the transition of HCV RNA detection for the 3 patients with virological breakthrough detected by CAM. Of the 13 patients with virological breakthrough detected by ART, none was positive for serum HCV RNA by CAM after week 24. This suggests that ART is more useful than CAM for monitoring virological breakthrough. To the contrary, of the 3 patients with virological breakthrough detected by CAM, 2 (66.7%) were positive for serum HCV RNA by ART during the treatment. For the other patient, HCV viremia was not detected only at weeks 36 and 48 by ART, which suggests that patients who had been thought to have relapsed might actually have continually had HCV viremia.

## Discussion

As part of the treatment strategy for chronic hepatitis C, measurement of the serum HCV RNA level is absolutely necessary because the earlier HCV RNA becomes

undetectable, the higher the probability of SVR [11, 15, 16, 26]. Therefore, the accuracy, sensitivity, lower limit of detection, and a broad dynamic range to monitor viral load change during antiviral therapy are important for the measurement of viremia. In the present study, we compared ART, a RealTime PCR assay, to CAM, the conventional method, and the results showed that ART was superior to CAM for monitoring viremia during antiviral therapy and more useful for the early prediction of virological response.

This study showed that the logarithmic declines of HCV RNA levels in SVR patients at all time points were significantly higher than those in non-SVR patients when measured with ART, but that the only significant difference was at week 4 when measured with CAM. These results indicate that ART is more useful than CAM for the prediction of SVR when patients have rapid declines of HCV RNA levels during the early phase of treatment. The reasons for this are that ART is an advanced quantitative assay based on the real-time PCR method, it is more sensitive, and has a broader range of determination than conventional methods [20, 27–29]. The lower limit of detection of ART is 12 IU/ml and the range of measurement is from 1.08 to 8.0 log IU/ml, but the detection limit of CAM is 500 IU/ml and the range of CAM is 2.7 to 6.7 log IU/ml.

PEG-IFN plus RBV treatment is currently the standard care for chronic hepatitis C patients, because it remarkably improves the SVR rate. However, there are still some patients who relapse during treatment and others who are negative at the end of the treatment but have flare-ups after the treatment. A suitable marker for the prediction of HCV viremia flare-up has not yet been identified. Minimal residual viremia detected by transcription-mediated amplification assay at the end of therapy in CAM-negative cases has been reported, which would be useful for predicting post-therapy relapse [30, 31]. Likewise, in the present study, all 13 patients with virological breakthrough detected by ART but not by CAM had relapsed after the treatment, and all 19 patients whose HCV RNA was undetectable at EOT by CAM but detectable by ART had relapsed. ART was superior to CAM for detecting virological breakthrough.

The differences in the limits of detection between the two assays would be responsible for this difference between ART and CAM in the virological dynamic change determined after the start of treatment. The principle of real-time PCR assays is to detect amplicon synthesis and deduce the number of viral genomes in the starting clinical sample during the PCR rather than at the end, and it is the reason that real-time PCR assays are more adequate for monitoring viral kinetics during antiviral treatment than end-point PCR assays [32].

Although there is a synergistic effect between IFN and RBV [33, 34], the logarithmic decline of serum HCV RNA induced by IFN in the early stages is significantly higher than that induced by RBV: IFN can induce several orders of logarithmic decline of serum HCV RNA, while RBV induces lower than 0.5 logarithmic decline [35, 36], so a more highly sensitive assay such as ART can detect virological breakthrough.

The results of the IDEAL (individualized dosing efficacy versus flat dosing to assess optional pegylated interferon therapy) trial showed that a logarithmic decline of HCV viremia at treatment week 4 of more than 3 log IU/ml or HCV viremia that becomes undetectable at week 12 are good indications of SVR [37, 38]. Our findings were similar to those of the IDEAL trial. Previously, it was recommended that antiviral therapy should be discontinued for patients whose HCV RNA levels had decreased to less than 2 log IU/ml at week 12 or those whose levels remained detectable after week 24 [15, 16]. The present study did not have such rules for stopping treatment. Moreover, most of our patients strongly hoped to complete the scheduled treatment even if they showed non-virological response. In fact, our previous multicenter study showed that only 5.9% of 273 patients, when given the option of stopping treatment because of non-virological response, stopped treatment [10]; thus, we had the data of such patients during the course of treatment and can corroborate such an application of the stopping rule.

We concluded that because of its accuracy and sensitivity for measuring HCV viremia, the Abbott RealTime PCR Assay is useful for monitoring HCV viremia during antiviral therapy and can be used for the prediction of SVR in patients chronically infected with HCV genotype 1.

## References

1. Hayashi J, Kishihara Y, Yamaji K, Furusyo N, Yamamoto T, Pae Y, et al. Hepatitis C viral quasispecies and liver damage in patients with chronic hepatitis C virus infection. Hepatology. 1997;25:697–701.
2. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264:1415–21.
3. Heim MH. Intracellular signaling and antiviral effects of interferons. Dig Liver Dis. 2000;32:257–63.
4. Frese M, Dazert E. Interferon-induced effector proteins and hepatitis C virus replication. In: Jirillo E, editor. Hepatitis C virus disease: immunobiology and clinical applications. USA: Springer; 2008. Chapter 6.
5. Chander G, Sulkowski MS, Jenckes MW, Torbenson MS, Herlong HF, Bass EB, et al. Treatment of chronic hepatitis C. A systematic review. Hepatology. 2002;36:S135–44.
6. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med. 1998;339:1485–92.
7. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomized trial of interferon alpha2b plus ribavirin for

48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet. 1998;352:1426–32.
8. Furusyo N, Katoh M, Tanabe Y, Kajiwara E, Maruyama T, Shimono J, et al. Interferon alpha plus ribavirin combination treatment of Japanese chronic hepatitis C patients with HCV genotype 2: a project of the Kyushu University Liver Disease Study Group. World J Gastroenterol. 2006;12:784–90.
9. Furusyo N, Kajiwara E, Takahashi K, Nomura H, Tanabe Y, Masumoto A, et al. Association between the treatment length and cumulative dose of pegylated interferon alpha-2b plus ribavirin and their effectiveness as a combination treatment for Japanese chronic hepatitis C patients : a project of the Kyushu University Liver Disease Study Group. J Gastroenterol Hepatol. 2008;23:1094–104.
10. Kainuma M, Furusyo N, Kajiwara E, Takahashi K, Nomura H, Tanabe Y, et al. Pegylated interferon α-2b plus ribavirin for older patients with chronic hepatitis C. World J Gastroenterol. 2010;16:4400–9.
11. Foster GR, Fried MW, Hadziyannis SJ, Messinger D, Freivogel K, Weiland O. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alpha-2a (40KD) and ribavirin. Scand J Gastroenterol. 2007;42:247–55.
12. Jensen DM, Morgan TR, Marcellin P, Pockros PJ, Reddy KR, Hadziyannis SJ, et al. Early identification of HCV genotype 1 patients responding to 24 weeks peginterferon alpha-2a (40 kd)/ ribavirin therapy. Hepatology. 2006;43:954–60.
13. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-α2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140:346–55.
14. National Institutes of Health. National Institutes of Health Consensus Development Conference Statement. Management of hepatitis C. Hepatology. 2002; 36:S3–20.
15. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, et al. Peginterferon alpha-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
16. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindoller R, et al. Peginterferon alpha-2b plus ribavirin compared with interferon alpha-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet. 2001;358:958–65.
17. Halfon P, Bourliere M, Penaranda G, Khiri H, Ouzan D, et al. Real-time PCR assays for hepatitis C virus (HCV) RNA quantitation are adequate for clinical management of patients with chronic HCV infection. J Clin Microbiol. 2006;44:2507–11.
18. Sabato MF, Shiffman ML, Langley MR, Wilkinson DS, Ferreira-Gonzalez A. Comparison of performance characteristics of three real-time reverse transcription-PCR test systems for detection and quantification of hepatitis C virus. J Clin Microbiol. 2007;45: 2529–36.
19. Schutten M, Peters D, Back NKT, Beld M, Beuselinck K, Foulongne V, et al. Multicenter evaluation of the new Abbott RealTime assays for quantitative detection of human immunodeficiency virus type 1 and hepatitis C virus RNA. J Clin Microbiol. 2007;45:1712–7.
20. Michelin BDA, Muller Z, Stelzl E, Marth E, Kessler HH. Evaluation of the Abbott RealTime HCV assay for quantitative detection of hepatitis C virus RNA. J Clin Microbiol. 2007;38:96–100.
21. Gerken G, Rothaar T, Rumi MG, Soffredini R, Trippler M, Blunk MJ, et al. Performance of the COBAS AMPLICOR HCV MONITOR test, version 2.0, an automated reverse transcription-PCR quantitative system for hepatitis C virus load determination. J Clin Microbiol. 2000;38:2210–4.
22. Konnick EQ, Erali M, Ashwood ER, Hillyard DR. Performance characteristics of the COBAS Amplicor Hepatitis C Virus (HCV) Monitor, Version 2.0, International Unit assay and the National Genetics Institute HCV Superquant assay. J Clin Microbiol. 2002;40:768–73.
23. Leckie G, Schneider G, Abravaya K, Hoenle R, Johanson J, Lampinen J, et al. Performance attributes of LCx HCV RNA quantitative assay. J Virol Methods. 2004;115:207–15.
24. Lee SC, Antony A, Lee N, Leibow J, Yang JQ, Soviero S, et al. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. J Clin Microbiol. 2000;38:4171–9.
25. Hayashi J, Ohmiya M, Kishihara Y, Tani Y, Kinukawa N, Ikematsu H, et al. A statistical analysis of predictive factors of response to human lymphoblastoid interferon in patients with chronic hepatitis C. Am J Gastroenterol. 1994;89:2151–6.
26. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology. 2003;38:645–52.
27. Pyne MT, Konnick EQ, Phansalkar A, Hillyard DR. Evaluation of the Abbott investigational use only RealTime hepatitis C virus (HCV) assay and comparison to the Roche TaqMan HCV analyte-specific reagent assay. J Clin Microbiol. 2009;47:2872–8.
28. Matsuura K, Tanaka Y, Hasegawa I, Ohno T, Tokuda H, Kurbanov F, et al. Abbott RealTime hepatitis C virus (HCV) and Roche Cobas AmpliPrep/Cobas TaqMan HCV assays for prediction of sustained virological response to pegylated interferon and ribavirin in chronic hepatitis C patients. J Clin Microbiol. 2009;47:385–9.
29. Fytili P, Tiemann C, Wang C, Schulz S, Schaffer S, Manns MP, et al. Frequency of very low HCV viremia detected by a highly sensitive HCV-RNA assay. J Clin Virol. 2007;39:308–11.
30. Ross RS, Viazov SO, Hoffmann S, Roggendorf M. Performance characteristics of a transcription-mediated nucleic acid amplification assay for qualitative detection of hepatitis C virus RNA. J Clin Lab Anal. 2001;15:308–13.
31. Gerotto M, Dal Pero F, Bortoletto G, Ferrari A, Pistis R, Sebastiani G, et al. Hepatitis C minimal residual viremia (MRV) detected by TMA at the end of Peg-IFN plus ribavirin therapy predicts post-treatment relapse. J Hepatol. 2006;44:83–7.
32. Pawlotsky JM. Molecular diagnosis of viral hepatitis. Gastroenterology. 2002;122:1554–68.
33. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature. 2005;436:967–72.
34. Suzuki T. A hepatitis C virus–host interaction involved in viral replicon: toward the identification of antiviral targets. Jpn J Infect Dis. 2010;63:307–11.
35. Pawlotsky JM, Dahari H, Neumann AU, Hezode C, Germanidis G, Lonjon I, et al. Antiviral action of ribavirin in chronic hepatitis C. Gastroenterology. 2004;126:703–14.
36. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, et al. Hepatitis C viral dynamics in vivo and the antiviral efficacy on interferon-alpha therapy. Science. 1998;282:103–7.
37. Sulkowski M, Vierling JM, Brown KA, Flamm SL, Kwo P, Mullen KD, et al. Probability of sustained virologic response (SVR) is associated with the magnitude of HCV RNA reduction at week 4 of treatment with peginterferon (PEG) plus ribavirin (RBV): results of the IDEAL trial. Hepatology. 2008;48:1144A (abstract).
38. Nyberg LM, Shiffman ML, Bonilla H, Hu KQ, Morgan TR, Levine RA, et al. Predicting the ability to achieve a sustained virologic response (SVR) in the first 12 weeks: results from the IDEAL study. Hepatology. 2008;48:1134A (abstract).

Hepatology Research

*Hepatology Research* 2011; 41: 1178–1188 doi: 10.1111/j.1872-034X.2011.00869.x

*Original Article*

# Longitudinal assessment of liver stiffness by transient elastography for chronic hepatitis B patients treated with nucleoside analog

Eiichi Ogawa,[1] Norihiro Furusyo,[1,2] Masayuki Murata,[1] Hachiro Ohnishi,[1] Kazuhiro Toyoda,[1] Hiroaki Taniai,[1] Takeshi Ihara,[2] Hiroaki Ikezaki,[2] Takeo Hayashi,[2] Mosaburo Kainuma[1] and Jun Hayashi[1,2]

[1]*Department of General Internal Medicine, Kyushu University Hospital, and* [2]*Department of Environmental Medicine and Infectious Disease, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan*

*Aim:* To evaluate the association between liver stiffness measured by transient elastography (FibroScan) and the efficacy of long-term nucleoside analog (NA) treatment for patients with chronic hepatitis B.

*Methods:* Study 1: Forty-four chronic HBV patients had liver stiffness measured by FibroScan and underwent liver biopsy. Study 2: Group A: 22 patients started NA treatment at entry and FibroScan was done annually for 3 years. Group B: 23 patients started NA treatment prior to pretreatment FibroScan measurement, and FibroScan was done for from 3 to 5 years after the start of NA treatment.

*Results:* Study 1: The FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$). Optimal cutoff of FibroScan values were 6.1 kPa for ≥ F1, 6.3 kPa for ≥ F2, 8.9 kPa for ≥ F3 and 12.0 kPa for F4. Study 2: For Group A, the baseline median FibroScan value was 8.2 kPa. FibroScan values significantly decreased annually for 3 years after the start of NA treatment (6.4 kPa, 5.8 kPa and 5.3 kPa at years 1, 2 and 3, respectively). For Group B, the FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

*Conclusions:* Liver stiffness, measured by transient elastography, of chronic hepatitis B patients treated with NA showed a rapid decline in the first 3 years followed by a more steady transition for from 3 to 5 years irrespective of long term virological effect.

**Key words:** breakthrough hepatitis, hepatitis B virus, liver fibrosis, nucleoside analog, transient elastography

## INTRODUCTION

CHRONIC HEPATITIS B virus (HBV) infection is a main cause of viral hepatitis. It is estimated that more than 350 million people are infected with HBV worldwide.[1,2] Morbidity and mortality by chronic HBV infection are a major public health concern. Lamivudine (LMV), an oral cytosine nucleoside analog (NA), was introduced for the treatment for HBV infection in 1998.[3,4] LMV can provide suppression of viral replication and biochemical improvement, which reduces the risk of developing serious liver diseases such as cirrhosis and hepatocellular carcinoma (HCC).[5–7]

*Correspondence: Dr Norihiro Furusyo, Department of General Internal Medicine, Kyushu University Hospital, Higashi-Ku, Fukuoka, 812-8582, Japan. Email: furusyo@gim.med.kyushu-u.ac.jp*
*Received 22 May 2011; revision 23 June 2011; accepted 5 July 2011.*

Although LMV has been shown to be highly effective in inhibiting HBV replication, the incidence of LMV-resistant virus is high, occurring in approximately 24% of patients after one year of treatment and in as many as 70% of patients after 4 years of treatment.[8] The emergence of LMV-resistance owing to the emergence of genotypic resistance by tyrosine, methionine, aspartate, or aspartate (YMDD) mutation may lead to viral and biochemical breakthrough and sometimes hepatitis flare-ups and rapid decompensation.[9] Adefovir dipivoxil (ADV) has been introduced for the treatment of chronic HBV infection, and ADV as an add-on to LMV has been used to reduce breakthrough.

Liver biopsy was for many years the gold standard for staging fibrosis. However, liver biopsy is no longer considered a perfect methodology because of the invasive nature of the procedure, sampling error, and inter-observer variability,[10] making improved testing

strategies necessary for accurate assessment of the liver status of patients with liver diseases. Recently, transient elastography (FibroScan; Echosens, Paris, France) has been proposed as a reliable, rapid, noninvasive and reproducible method for measuring liver stiffness.[11] FibroScan is increasingly being used as a noninvasive method for assessing liver fibrosis, and there have been many reports to date on patients with various liver diseases, such as chronic hepatitis C, primary biliary disease, non-alcoholic steatohepatitis, and chronic hepatitis B.[12–14] However, there are few reports of an association between the values as measured by FibroScan (FibroScan values) and the effectiveness of treatments for chronic liver diseases. We previously showed that FibroScan values were significantly correlated with the histological stage of percutaneous liver biopsy of patients with chronic hepatitis B and C.[15] Longitudinal assessment by FibroScan among patients with chronic hepatitis C treated with pegylated interferon alpha-2b and ribavirin was also done, with FibroScan shown to be a useful tool for the diagnosis of liver fibrosis and follow-up assessment of antiviral treatment.[16]

The aims of the present study were as follows: (Study 1) to test the correlation between liver histology and liver stiffness measured by FibroScan before NA treatment and (Study 2) to evaluate the association between liver stiffness measured by FibroScan and the efficacy of NA treatment for long-term in chronic HBV patients.

## METHODS

### Patients

IN STUDY 1, to evaluate the relationship between histological findings and FibroScan values, the liver stiffness of 44 patients with chronic HBV infection was measured by FibroScan and the patients underwent liver biopsy around the same time. The baseline laboratory results of the study population are summarized in Table 1.

In Study 2, 45 patients with chronic HBV infection undergoing long-term NA treatment had annual measurements of liver elasticity by FibroScan. These patients were divided into two groups according to the time of initiation of NA treatment: Since 2005 and between 2001 and 2004 (Groups A and B, respectively). In Group A, 22 patients started NA treatment at entry and FibroScan was done annually for 3 years (17 patients included in Study 1). In Group B, 23 patients started NA treatment prior to our pretreatment FibroScan measurement program, so FibroScan was done from 3 to 5 years after the start of NA treatment. The baseline laboratory results of the study population are summarized in Table 2.

**Table 1** Baseline of characteristics of patients with chronic hepatitis B virus (HBV) infection (Study 1)

| Characteristics | Liver biopsy $n = 44$ |
|---|---|
| Male (%) | 29 (64.4) |
| Age (years) | 47.0 ± 13.9 |
| Alanine aminotransferase (IU/L) | 50.4 ± 29.0 |
| Platelet count ($10^9$/L) | 163 ± 53 |
| Prothrombin time (%) | 90.6 ± 11.3 |
| α-fetoprotein (ng/mL) | 5.5 ± 10.9 |
| Serum type IV collagen (ng/mL) | 165 ± 81 |
| HBeAg positive No. (%) | 19 (42.2) |
| Serum HBV DNA level (Log copies/mL)† | 5.3 ± 1.5 |
| FibroScan values (kPa) | 6.3 (3.3–25.7) |
| Liver histology | |
| Stage of fibrosis (F0/F1/F2/F3/F4) | 6/18/12/4/4 |
| Grade of activity (A0/A1/A2/A3) | 0/19/23/2 |

†Logarithmic transformed copies/mL.
Data is shown by the mean ± standard deviation, median (range) or *n* (%).
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NA, nucleoside analog.

All patients satisfied the following criteria: (1) positive for hepatitis B surface antigen and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Exclusion criteria for the study were: (1) positive for antibody to human immunodeficiency virus or positive for anti-hepatitis C virus; (2) clinical or biochemical evidence of hepatic decompensation; (3) excessive active alcohol consumption (> 60 g/day converted into ethanol) or drug abuse; (4) suspected hepatocellular carcinoma at entry; or (5) treatment with immunosuppressive agents within 12 months prior to enrollment. Patients who fulfilled the above criteria were recruited for treatment at Kyushu University Hospital.

Informed consent was obtained from all patients before enrollment. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

### Clinical and laboratory assessment

Clinical parameters included ALT, platelet count, α-fetoprotein (AFP), type IV collagen, prothrombin time (PT), HBV genotype and HBV DNA. Body mass index (BMI) was calculated as weight in kilograms/height in square meters. Serum levels of ALT, platelet

**Table 2** Baseline (proior to NA treatment) of characteristics of patients with chronic hepatitis B virus (HBV) infection (Study 2)

| Characteristics | Group A $n = 22$ | Group B $n = 23$ | *P*-values |
|---|---|---|---|
| Male (%) | 14 (63.6) | 16 (69.6) | 0.4567 |
| Age (years) | 49.8 ± 8.1 | 50.7 ± 10.3 | 0.7652 |
| Body mass index (kg/m$^2$) | 22.9 ± 2.5 | 22.4 ± 2.5 | 0.1786 |
| Alanine aminotransferase (IU/L) | 54.6 ± 30.4 | 56.1 ± 28.3 | 0.8613 |
| Platelet count (10$^9$/L) | 137 ± 54 | 156 ± 51 | 0.2128 |
| Prothrombin time (%) | 88.1 ± 11.2 | 80.2 ± 9.9 | 0.0239 |
| α-fetoprotein (ng/mL) | 5.0 ± 3.0 | 5.5 ± 4.0 | 0.6135 |
| Serum type IV collagen (ng/mL) | 172 ± 58 | Not evaluated | – |
| HBeAg positive No. (%) | 7 (31.8) | 7 (30.4) | 0.9202 |
| Serum HBV DNA level (Log copies/mL)† | 5.9 ± 1.5 | 6.2 ± 1.3 | 0.4448 |
| FibroScan values (kPa) | 8.2 (4.2–28.5) | Not evaluated | – |
| Liver histology | | | |
| Stage of fibrosis (F0/F1/F2/F3/F4) | 2/5/7/1/2 | 0/3/5/7/5 | 0.1051 |
| Grade of activity (A0/A1/A2/A3) | 0/3/13/1 | 0/3/13/4 | 0.5166 |
| Not determined (*n*) | 5 | 3 | |

†Logarithmic transformed copies/mL.
Data is shown by the mean ± standard deviation, median (range), or *n* (%).
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NA, nucleoside analog.

count, AFP, type IV collagen and PT were measured by standard laboratory techniques at a commercial laboratory (MBC Laboratory, Tokyo, Japan).

## HBV genotyping and HBV DNA measurement

Hepatitis B virus genotyping was determined serologically by the polymerase chain reaction (PCR)-invader method with genotype specific probes.[17] Quantification of serum HBV DNA was performed by quantitative PCR assay (Amplicor HBV Monitor, Roche Diagnostics, Mannheim, Germany), over a detection range from 2.6 (corresponding to 400 copies/mL) to 7.5 log copies/mL. Virological breakthrough was defined as the reappearance of serum HBV DNA to a level more than 10-fold (1 log) the minimum during treatment.[18]

## Monitoring the emergence of LMV-resistant mutants

The emergence of LMV-resistant mutants is mainly based on point mutation from methionine to valine/isoleucine at rt204 (rt204V/L) in the YMDD motif. YMDD mutation was detected by rapid PCR amplification across the YMDD-encoding gene locus and analysis of the hybridization kinetics of an integrated probe to infer its sequence using the LightCycler (Roche Diagnostics).[19,20]

## Nucleoside analog treatment

Of the 45 patients treated with NA, 38 (84.4%) received LMV (Zeffix; Glaxo Smith Kline, UK) in a single oral daily dose of 100 mg and the other seven (15.6%) received entecavir (ETV) (Baraclude; Bristol-Myers Squibb, USA) in a single oral daily dose of 0.5 mg. Breakthrough hepatitis (BTH) by drug-resistant YMDD mutants developed in 13 (28.9%) of these patients. BTH patients received 10 mg ADV (Hepsera; Glaxo Smith Kline) in addition to LMV daily.

## Transient elastography (FibroScan)

FibroScan was done for the right lobe of the liver through the intercostal spaces with the patient lying in the dorsal decubitus position with the right arm in maximal position. The tip of the probe transducer was covered with coupling gel and placed on the skin between the ribs at the level of the right lobe of the liver. The operator, assisted by an ultrasonic time-motion image, located a liver portion at least 6 cm thick and free of large vascular structures. Once the measurement area had been located, the operator pressed the probe button to start acquisition. The elasticity was automatically calculated by the apparatus, with the data shown as kilopascal (kPa). All examinations were performed by accomplished operators of our department. Only liver stiffness measurements

**Table 3** Optimal cutoff of FibroScan values for the determination of histological fibrosis stage in 44 biopsy-received patients with chronic hepatitis B virus (HBV) infection (Study 1)

| | Histological fibrosis stage by liver biopsy | | | |
|---|---|---|---|---|
| | F ≥ 1 | F ≥ 2 | F ≥ 3 | F = 4 |
| Cutoff value* (kPa) | 6.1 | 6.3 | 8.9 | 12.0 |
| AUROC | 0.67 | 0.86 | 0.87 | 0.89 |
| Sensitivity (%) | 65.9 | 95.2 | 87.5 | 75.0 |
| Specificity (%) | 71.4 | 74.0 | 75.0 | 88.6 |
| Positive predictive value (%) | 93.1 | 74.1 | 41.2 | 37.5 |
| Negative predictive value (%) | 26.3 | 95.2 | 96.8 | 97.5 |
| Positive likelihood ratio | 2.30 | 3.66 | 3.50 | 6.58 |

*The optimal cutoff value is the one that gives the higher total sensitivity and specificity.
AUROC, area under the receiver operating characteristic curve.

obtained with at least six successful acquisitions and a success rate of at least 60% were considered reliable. The validity of FibroScan values depends on an interquartile range of all successful measurements (IQR/M) of less than 30% of median values.[21] The mean IQR/M of the present study was 22.1% and no cases of IQR/M > 30% were found.

### Liver histology and quantification of liver biopsy

Liver biopsy was performed by experienced hepatologists with a 16G disposable needle (Bard Monopty; C.R.Bard, Covington, USA) under ultrasound guidance. The median liver biopsy length was 18 mm (minimum length 15 mm). Liver biopsy specimens were fixed in formalin and paraffin was embedded. All biopsy specimens were analyzed by two experienced pathologists who were blinded to the clinical data. For each specimen, the stage of fibrosis and the grade of activity were established according to the METAVIR score.[22] Fibrosis was staged on a 0–4 scale as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis and few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity.

### Breakthrough hepatitis

Biochemical breakthrough usually lags behind virological breakthrough, and serum ALT level may remain normal for weeks to years after the development of antiviral resistance. BTH was defined as a serum ALT level over five times the upper limit of the normal range.

### Statistical analysis

Statistical analysis was done with biomedical computer programs (BMDP) statistical software for the IBM 3090 system computer (BMBD Statistical Software, Inc., Los Angeles, CA, USA). Continuous data were expressed as median or mean ± standard deviation (SD). The paired *t*-test, unpaired *t*-test, Mann-Whitney *U*-test or Kruskal–Wallis non-parametric analysis of variance (ANOVA) was used for the analysis. Area under the receiver operating characteristic curve (AUROC) analysis was done to evaluate the relationship between histological findings and FibroScan values. The cutoff values were selected from the receiver operating characteristic curve to maximize the total sensitivity and specificity. A *P*-value less than 0.05 was regarded as statistically significant.

## RESULTS

### Relationship between liver fibrosis and FibroScan values at baseline

ALL PATIENTS WERE infected with HBV genotype C. The median values (interquartile range) of the patients were 5.0 kPa (3.5–6.0), 5.7 kPa (5.0–6.0), 7.0 kPa (6.2–10.1), 9.4 kPa (9.2–13.3) and 18.0 kPa (12.9–21.9) for F0, F1, F2, F3 and F4, respectively. The FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$) and were also significantly increased in accordance with the grade of activity of the patients ($r = 0.321$, $P < 0.0001$). According to the progression of liver fibrosis stage, the mean grade of activity was higher (F0: 1.2, F1: 1.4, F2 1.8, F3 2.0 and F4 2.0), but the analysis did not reach significance. Table 3 shows the optimal liver stiffness cutoff values obtained by sensitivity, specificity and positive

likelihood ratios. Four threshold FibroScan values were identified: 6.1 kPa for ≥F1 (sensitivity 65.9%, specificity 71.4%); 6.3 kPa for ≥F2 (sensitivity 95.2%, specificity 74.0%); 8.9 kPa for ≥F3 (sensitivity 87.5%, specificity 75.0%) and 12.0 kPa for F4 (sensitivity 75.0%, specificity 88.6%). The corresponding AUROC were 0.67 for ≥F1, 0.86 for ≥F2, 0.87 for ≥F3 and 0.89 for F = 4.

### Longitudinal FibroScan values 1–3 years after the start of the nucleoside analog treatment (Group A, Tables 4,6)

All patients were infected with HBV genotype C. The clinical and FibroScan data of Group A are shown in Table 4. For 19 patients (86.4%) the serum HBV DNA level became undetectable and that of the other three patients was reduced to under 4.0 log copies/mL on PCR by 6 months after the start of NA treatment. ALT level (54.6 ± 30.4 to 22.1 ± 7.7 U/L, $P < 0.0001$), PT (88.1 ± 11.2 to 96.3 ± 9.1%, $P < 0.0001$), AFP (5.0 ± 3.0 to 3.0 ± 1.6 ng/mL, $P = 0.0004$) and serum type IV collagen (172 ± 58 to 142 ± 37 ng/mL, $P = 0.0023$) were significantly improved during the first 3 years of NA treatment. The baseline median FibroScan value was 8.2 kPa and the mean value was 10.5 ± 6.5 kPa in Group A. FibroScan values significantly decreased annually for 3 years after the start of NA treatment (median value 6.4 kPa [mean value 8.7 ± 5.6 kPa], 5.8 kPa [7.4 ± 4.6 kPa] and 5.3 kPa [6.8 ± 4.0 kPa] at years 1, 2 and 3, respectively).

For non-BTH patients ($n = 18$, 81.8%), the baseline median FibroScan value was 7.7 kPa and the mean value was 10.3 ± 7.0 kPa. FibroScan values showed a significant, annual decrease for 3 years after the start of NA treatment (median value 6.3 kPa [mean value 8.4 ± 5.9 kPa], 5.4 kPa [7.2 ± 5.0 kPa] and 5.0 kPa [6.5 ± 4.3 kPa] at years 1, 2 and 3, respectively). For patients with an episode of BTH and viral breakthrough due to YMDD mutation, the serum HBV DNA level became undetectable on PCR by 6 months after the additional medication (ADV added-on LMV). For BTH patients ($n = 4$, 18.2%), the baseline median FibroScan value was 10.2 kPa and the mean value was 11.2 ± 4.3 kPa. FibroScan values improved due to NA treatment for the first 2 years (median value 10.8 kPa [mean value 10.2 ± 3.9 kPa], 7.9 kPa [7.9 ± 2.4 kPa] and 8.2 kPa [8.0 ± 2.2 kPa] at years 1, 2 and 3, respectively). However, three of four patients had BTH occur in the 3 year, therefore, FibroScan values did not improve.

### Longitudinal FibroScan values 3–5 years after the start of nucleoside analog treatment (Study 2: Group B, Tables 5,6)

The clinical and FibroScan data of Group B are shown in Table 5. The serum HBV DNA level of 20 patients (87.0%) became undetectable and that of the other three patients was under 4.0 log copies/mL by PCR by 6 months after the start of treatment. For patients with an episode of BTH or viral breakthrough due to YMDD mutation, the serum HBV DNA level became undetectable by PCR by 6 months after the addition of ADV to LMV. Neither liver fibrosis nor the biochemical markers (ALT: 25.2 ± 16.8 to 24.2 ± 13.8 IU/L, PT: 91.1 ± 10.8 to 92.7 ± 8.7%, AFP: 3.1 ± 2.1 to 3.3 ± 3.1 ng/mL and serum type IV collagen: 154 ± 51 to 148 ± 48 ng/mL) of patients with or without BTH statistically improved over the 3 years after the start of NA treatment. At 3, 4 and 5 years after the start of NA treatment, the median (mean) FibroScan values were 6.1 (8.1 ± 5.2) kPa, 6.7 (8.2 ± 5.2) kPa and 5.9 (8.1 ± 5.1) kPa, respectively. For BTH patients ($n = 9$, 39.1%) at 3, 4 and 5 years after the start of NA treatment, the median (mean) FibroScan values were 10.4 (11.0 ± 6.4) kPa, 10.2 (11.1 ± 6.8) kPa and 9.6 (11.1 ± 6.6) kPa, respectively. Similarly, for non-BTH patients ($n = 14$, 60.9%), the median (mean) FibroScan values were 5.2 (6.2 ± 3.3) kPa, 5.2 (6.3 ± 2.8) kPa and 5.3 (6.3 ± 2.7) kPa, respectively. In both the BTH and non-BTH groups, FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

### Relationship between hepatocellular carcinoma and breakthrough hepatitis

Three of the patients (13.6%) in Group A and three (13.0%) in Group B developed HCC in the follow-up period: Four (66.7%) had FibroScan values consistently over 10 kPa. Patients no. 4 in Group A and no. 20 in Group B developed HCC after the start of NA treatment, in spite of low FibroScan values. Both of these patients had YMDD mutation detected by PCR and an elevated HBV DNA level before the occurrence of HCC.

In analysis of the relation between HCC and BTH, the incidence of HCC for BTH patients (4 of 13, 30.8%) was significantly higher than that of non-BTH patients (2 of 32, 6.3%) ($P = 0.0332$).

## DISCUSSION

THIS STUDY DEMONSTRATED an association between liver stiffness measured by FibroScan and the efficacy of NA treatment of patients with chronic

Table 4 Clinical data and longitudinal FibroScan values in Group A (Study 2)

| Patient No. | Age (years) | Sex | BMI (kg/m²) | Histology | | FibroScan values (kPa) | | | | ALT (IU/L) | | | | HBeAg | HBV DNA (LogIU/mL) | | | | BTH | HCC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | F-stage | A-grade | FS-0 | FS-1 | FS-2 | FS-3 | ALT-0 | ALT-1 | ALT-2 | ALT-3 | | DNA-0 | DNA-1 | DNA-2 | DNA-3 | | |
| 1 | 48 | M | 22.5 | 2 | 2 | 17.3 | 14.0 | 12.9 | 12.6 | 53 | 30 | 35 | 29 | – | 5.5 | – | – | 4.2 | + (3) | + (3) |
| 2 | 56 | F | 21.1 | not tested | not tested | 10.4 | 9.6 | 7.9 | 8.6 | 27 | 18 | 16 | 13 | + | 6.8 | – | – | 5.4 | + (3) | – |
| 3 | 53 | M | 23.0 | 2 | 2 | 9.9 | 12.0 | 7.9 | 7.7 | 67 | 84 | 20 | 19 | + | 7.5 | 5.9 | – | – | + (1) | – |
| 4 | 42 | M | 23.4 | not tested | not tested | 7.1 | 5.1 | 5.0 | 5.2 | 44 | 23 | 20 | 21 | + | 7.7 | 3.2 | – | 5.4 | + (3) | + (3) |
| 5 | 42 | F | 21.4 | 0 | 2 | 4.2 | 4.0 | 3.8 | 2.5 | 25 | 14 | 15 | 16 | – | 5.5 | – | – | – | – | – |
| 6 | 65 | M | 26.3 | 1 | 1 | 5.2 | 4.3 | 4.0 | 3.4 | 35 | 17 | 20 | 34 | – | 3.4 | – | – | 3.0 | – | – |
| 7 | 34 | M | 24.2 | 1 | 2 | 5.4 | 4.8 | 3.8 | 3.9 | 74 | 36 | 45 | 36 | + | 7.5 | – | – | – | – | – |
| 8 | 56 | F | 23.6 | 0 | 1 | 5.5 | 4.8 | 4.7 | 4.0 | 87 | 17 | 15 | 16 | – | 7.0 | – | – | – | – | – |
| 9 | 57 | M | 22.5 | not tested | not tested | 5.6 | 4.8 | 4.0 | 4.0 | 43 | 37 | 35 | 32 | – | 7.3 | – | – | – | – | – |
| 10 | 51 | M | 28.6 | 1 | 2 | 5.8 | 6.2 | 5.6 | 5.6 | 55 | 28 | 27 | 21 | – | 5.4 | – | – | – | – | – |
| 11 | 49 | F | 22.9 | 2 | 3 | 7.0 | 5.3 | 4.6 | 2.9 | 59 | 18 | 12 | 12 | + | 6.2 | – | – | – | – | – |
| 12 | 32 | F | 29.1 | 1 | 2 | 7.2 | 5.4 | 5.2 | 4.8 | 65 | 28 | 27 | 27 | – | 4.8 | – | – | – | – | – |
| 13 | 37 | M | 22.3 | 1 | 1 | 7.5 | 6.4 | 6.1 | 5.2 | 99 | 17 | 12 | 18 | + | 7.7 | 3.4 | – | – | – | – |
| 14 | 52 | M | 23.8 | 3 | 2 | 7.9 | 5.2 | 3.9 | 4.8 | 96 | 17 | 19 | 11 | – | 4.1 | – | – | – | – | – |
| 15 | 55 | M | 22.5 | not tested | not tested | 8.5 | 6.3 | 6.0 | 5.8 | 47 | 26 | 25 | 35 | – | 7.3 | – | – | – | – | – |
| 16 | 55 | M | 20.8 | 2 | 2 | 8.6 | 7.4 | 6.2 | 5.3 | 42 | 20 | 25 | 27 | – | 5.0 | – | – | – | – | – |
| 17 | 51 | F | 21.1 | 2 | 2 | 9.4 | 7.4 | 5.9 | 5.4 | 36 | 29 | 23 | 24 | – | 6.1 | – | – | – | – | – |
| 18 | 48 | M | 23.9 | 2 | 2 | 12.8 | 7.2 | 5.2 | 4.8 | 45 | 22 | 29 | 22 | – | 5.5 | – | – | – | – | – |
| 19 | 53 | M | 21.6 | 4 | 2 | 13.3 | 11.5 | 9.6 | 8.6 | 13 | 14 | 12 | 12 | – | 3.7 | – | – | – | – | – |
| 20 | 56 | F | 22.7 | 2 | 2 | 17.3 | 14.2 | 10.9 | 10.4 | 21 | 16 | 18 | 20 | – | 4.5 | – | – | – | – | – |
| 21 | 55 | F | 21.2 | 4 | 2 | 25.7 | 22.1 | 17.6 | 15.1 | 141 | 29 | 21 | 24 | – | 6.0 | – | 2.7 | 3.0 | – | + (3) |
| 22 | 49 | M | 27.5 | not tested | not tested | 28.5 | 24.0 | 21.2 | 18.0 | 27 | 19 | 20 | 17 | – | 3.4 | – | – | – | – | – |
| | | | | | median | 8.2 | 6.4 | 5.8 | 5.3 | | | | | | | | | | | |

FS-0, FibroScan values at baseline; FS-1, 2 and 3, FibroScan values at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
M, male; F, female; BMI, body mass index; F-stage, fibrosis stage; A-grade, activity grade; ALT, alanine aminotranseferase; BTH, breakthrough hepatitis; HCC, hepatocellular carcinoma.
ALT-0, ALT level at baseline; ALT-1, 2 and 3, ALT levels at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
DNA-0, HBV DNA level at baseline; DNA-1, 2 and 3, HBV DNA levels at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
Figure in parenthesis was the onset of BTH or HCC after the start of nucleoside analog treatment (unit: year).

**Table 5** Clinical data and longitudinal FibroScan values in Group B (Study 2)

| Patient No. | Age (years) | Sex | BMI (kg/m$^2$) | Histology | | FibroScan values (kPa) | | | ALT (IU/L) | | | HBeAg | HBV DNA (LogIU/mL) | | | | | | BTH | HCC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | F-stage | A-grade | FS-3 | FS-4 | FS-5 | ALT-0 | ALT-3 | ALT-5 | | DNA-0 | DNA-1 | DNA-2 | DNA-3 | DNA-4 | DNA-5 | | |
| 1 | 64 | M | 22.7 | 4 | 3 | 20.5 | 23.3 | 21.8 | 34 | 14 | 16 | – | 7.1 | – | 4.9 | – | – | – | + (2) | + (2) |
| 2 | 50 | M | 24.4 | 4 | 2 | 20.4 | 17.7 | 18.8 | 83 | 29 | 25 | + | 6.5 | – | 3.9 | 3.6 | 3.6 | 2.8 | + (2) | – |
| 3 | 56 | M | 23.7 | 3 | 2 | 14.3 | 16.7 | 16.6 | 24 | 17 | 15 | – | 6.5 | – | – | 5.3 | – | 2.7 | + (3) | + (5) |
| 4 | 61 | F | 24.2 | 3 | 3 | 10.6 | 8.5 | 9.6 | 56 | 38 | 41 | + | 7.8 | – | 6.8 | 3.1 | – | – | + (2) | – |
| 5 | 46 | F | 22.0 | 4 | 3 | 10.4 | 10.2 | 9.6 | 63 | 27 | 24 | – | 7.1 | – | – | – | 4.0 | – | + (4) | – |
| 6 | 43 | M | 22.2 | 4 | 3 | 9.5 | 10.2 | 9.8 | 56 | 38 | 41 | + | 4.1 | 6.0 | – | – | – | – | + (1) | – |
| 7 | 49 | M | 21.5 | 3 | 2 | 6.5 | 6.7 | 6.4 | 32 | 24 | 22 | – | 6.0 | – | – | – | 4.0 | – | + (4) | – |
| 8 | 47 | M | 21.5 | 1 | 1 | 3.7 | 3.5 | 4.0 | 31 | 29 | 29 | – | 6.4 | – | – | – | – | 3.9 | + (5) | – |
| 9 | 57 | M | 20.7 | 3 | 2 | 3.2 | 3.5 | 3.0 | 40 | 14 | 12 | + | 8.6 | – | 7.1 | 4.0 | – | – | + (2) | – |
| 10 | 71 | F | 22.6 | 1 | 1 | 3.9 | 4.3 | 5.1 | 25 | 13 | 11 | – | 5.5 | – | – | – | – | – | – | – |
| 11 | 35 | M | 22.6 | 2 | 2 | 4.4 | 5.0 | 5.0 | 97 | 15 | 14 | + | 7.6 | – | – | – | – | – | – | – |
| 12 | 55 | M | 22.4 | 1 | 1 | 4.6 | 4.5 | 3.8 | 42 | 17 | 15 | – | 3.3 | – | – | – | – | – | – | – |
| 13 | 60 | F | 19.1 | not tested | not tested | 4.8 | 3.8 | 4.8 | 77 | 13 | 11 | – | 6.0 | – | – | – | – | – | – | – |
| 14 | 47 | M | 24.2 | 2 | 2 | 4.8 | 5.2 | 4.3 | 61 | 41 | 47 | – | 6.3 | – | – | – | – | – | – | – |
| 15 | 58 | F | 21.2 | 2 | 2 | 4.8 | 5.2 | 5.2 | 38 | 10 | 14 | – | 7.0 | – | – | – | 3.7 | 3.9 | – | – |
| 16 | 60 | M | 24.0 | 3 | 2 | 5.1 | 5.8 | 5.4 | 119 | 26 | 24 | – | 4.6 | – | 3.0 | 2.7 | 3.1 | 3.0 | – | – |
| 17 | 41 | F | 18.9 | 2 | 2 | 5.3 | 4.8 | 5.9 | 39 | 7 | 13 | + | 7.6 | – | – | – | – | – | – | – |
| 18 | 44 | M | 20.9 | not tested | not tested | 5.5 | 6.8 | 5.8 | 23 | 11 | 17 | – | 5.6 | – | – | – | – | – | – | – |
| 19 | 33 | M | 21.3 | 2 | 2 | 6.1 | 5.0 | 4.8 | 73 | 41 | 41 | + | 7.2 | 2.7 | 3.3 | – | – | – | – | – |
| 20 | 51 | M | 23.6 | 3 | 2 | 6.1 | 6.8 | 6.1 | 54 | 29 | 27 | – | 6.1 | – | 3.6 | 3.0 | 3.8 | 3.2 | – | + (2) |
| 21 | 43 | F | 18.2 | not tested | not tested | 6.6 | 6.8 | 7.5 | 73 | 32 | 27 | – | 6.5 | – | – | – | – | – | – | – |
| 22 | 62 | M | 23.6 | 4 | 2 | 8.3 | 10.5 | 10.1 | 33 | 28 | 26 | – | 3.8 | – | – | – | – | – | – | – |
| 23 | 32 | M | 30.3 | 3 | 2 | 17.1 | 14.0 | 13.9 | 118 | 87 | 68 | – | 6.1 | – | – | – | – | 3.3 | – | – |
| | | | | | median | 6.1 | 6.7 | 5.9 | | | | | | | | | | | | |

FS-3, 4 and 5, FibroScan values at 3, 4 and 5 years after the start of nucleoside analog treatment, respectively.
M, male; F, female; BMI, body mass index; F-stage, fibrosis stage; A-grade, activity grade; ALT, alanine aminotransferase; BTH, breakthrough hepatitis; HCC, hepatocellular carcinoma.
ALT-0, ALT level at baseline; ALT-3 and 5, ALT levels at 3 and 5 years after the start of nucleoside analog treatment, respectively.
DNA-0, HBV DNA level at baseline; DNA-1, 2, 3, 4 and 5, HBV DNA levels at 1, 2, 3, 4 and 5 years after the start of nucleoside analog, respectively.
Figure in parenthesis was the onset of BTH or HCC after the start of nucleoside analog treatment (unit: year).

**Table 6** Longitudinal assessment of FibroScan values and biochemical parameters after the start of nucleoside analog treatment (Study 2)

| | FibroScan (kPa) | *P*-value | ALT (IU/L) | *P*-value | PT (%) | *P*-value | AFP (ng/mL) | *P*-value | IV-C (ng/mL) | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|
| Group A | | | | | | | | | | |
| All patients ($n = 22$) | | | | | | | | | | |
| Baseline | 8.2 (4.2–28.5) | | 54.6 ± 30.4 | | 88.1 ± 11.2 | | 5.0 ± 3.0 | | 172 ± 58 | |
| FS-1 | 6.4 (4.0–24.0) | <0.0001* | 25.4 ± 14.8 | 0.0002* | | | | | | |
| FS-2 | 5.8 (3.8–21.2) | <0.0001** | 22.3 ± 8.3 | 0.3211** | | | | | | |
| FS-3 | 5.3 (2.5–18.0) | 0.0064*** | 22.1 ± 7.7 | 0.8494*** | 96.3 ± 9.1 | <0.0001 | 3.0 ± 1.6 | 0.0004 | 142 ± 37 | 0.0023 |
| Group B | | | | | | | | | | |
| All patients ($n = 23$) | | | | | | | | | | |
| Baseline | not tested | | 56.1 ± 28.3 | | 80.2 ± 9.9 | | 5.5 ± 4.0 | | not tested | |
| FS-3 | 6.1 (3.2–20.5) | | 25.2 ± 16.8 | 0.0001* | 91.1 ± 10.8 | 0.0001* | 3.1 ± 2.1 | 0.0004* | 154 ± 51 | |
| FS-4 | 6.7 (3.5–23.3) | 0.7439# | | | | | | | | |
| FS-5 | 5.9 (3.0–21.8) | 0.6785## | 24.2 ± 13.8 | 0.3530# | 92.7 ± 8.7 | 0.2459# | 3.3 ± 3.1 | 0.5401# | 148 ± 48 | 0.1884# |

*, compared to Baseline; **, ***, compared to FS-1; #, compared to FS-2; ##, compared to FS-3; ###, compared to FS-4.
Data are shown by the mean ± standard deviation or median (range).
FS-1, 2, 3, 4 and 5, FibroScan values at 1, 2, 3, 4 and 5 years after the start of nucleoside analog treatment, respectively.
ALT, alanine aminotransferase; PT, prothrombin time; AFP, α-fetoprotein; IV-C, type IV collagen.

hepatitis B. Recent reports showed that 12 months ETV treatment for chronic hepatitis B is associated with an improvement of FibroScan values.[23,24] The results suggested that a decrease in FibroScan values during the first year of NA treatment might be attributed to not only improvement of liver fibrosis but also to necroinflammation. We were able to investigate the association between the efficacy of NA treatment and FibroScan values for a much longer period than the previous reports. Suzuki *et al.*[25] found that a 3-year LMV therapy could induce histological improvements whether YMDD mutants accompanied by virological breakthrough and BTH appeared. Our findings also showed that liver stiffness markedly improved during the first 3 years of NA treatment, but that the degree of improvement was less after 3 years of NA treatment, irrespective of the long term virological effect.

First, we investigated the association between liver fibrosis by liver biopsy and FibroScan values. Liver biopsy is not always acceptable to patients and biochemical liver examinations are sometimes unreliable for determining the extent of liver fibrosis because patients who have advanced liver fibrosis may have normal ALT levels. We showed that the FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$). However, the cutoff for chronic hepatitis B (F3 8.9 kPa, F4 12.0 kPa) was lower than that of chronic hepatitis C (F3 10.3 kPa, F4 14.9 kPa)[16] especially for predicting severe fibrosis and cirrhosis. Macronodular cirrhosis, characterized by large nodules delimited by thin septa, is commonly found in patients with HBV infection. A different type and/or extent of liver inflammatory infiltrate within liver blocks, characterized by interface and/or lobular hepatitis infected with HBV and by the portal tract lymphoid aggregate near or surrounding bile ducts infected with HCV, might account for the difference of cutoff values between chronic hepatitis B and C patients.

Usually, complete long-term suppression of HBV DNA is an essential goal of treatment for chronic hepatitis B. Liver fibrosis is potentially reversible after viral replication has subsided,[26] therefore, the longitudinal assessment of liver fibrosis treated with NA is very important. We previously showed a dramatic reduction of FibroScan values for both virological and biochemical response by chronic hepatitis C patients treated with pegylated interferon alpha-2b and ribavirin treatment.[16] In this study of chronic hepatitis B patients who underwent NA treatment, the FibroScan values showed a rapid decline without BTH in the first 3 years. In the treatment of chronic hepatitis C, sustained virological clearance is

an ultimate aim. However, even successful response to NA by chronic hepatitis B patients does not bring about clearance. In fact, our data showed that in both the BTH and non-BTH groups, FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

Previous reports have shown a well-marked association between elevated HBV DNA and progression to cirrhosis and HCC.[27] Even when ALT levels were normal, HBeAg negative patients who had HBV DNA over 4.0 log copies/mL had an increased risk of developing liver cirrhosis or HCC. Although HBV genotypes also influence the pathological features of patients, the virologic and molecular mechanisms involved remain largely not understood. Epidemiologic studies have shown that each genotype has a distinct geographic and ethnic distribution.[28,29] HBV genotypes A and D occur frequently in Africa, Europe and India, while genotypes B and C are prevalent in Asia. In general, genotype C has been shown to be associated with more progressive hepatitis than genotype B.[30] Recently, the intracellular expression of HBV DNA and hepatitis B core protein were shown to be higher for genotypes B and C than for genotypes A and D in an experimental study.[31] The intracellular accumulation of HBV DNA and antigens may play a role in liver cell damage, and moreover, the higher replication capacity of genotype C may explain the association with more severe histological liver damage than is seen for other genotypes and continued higher FibroScan values than in the case of long term HCV infection, irrespective of well-controlled antiviral treatment.

The introduction of LMV has resulted in improved suppression of HBV replication, improving histological necroinflammation and fibrosis. However, LMV-resistant HBV (the emergence of YMDD mutation) has appeared with prolonged LMV treatment, which can lead to viral or biochemical breakthrough. Some patients with hepatic cirrhosis suffer severe deterioration after BTH. Among patients who received LMV and maintained an ALT level of under 40 IU/L, the rate of YMDD motif mutant and BTH occurrences were 11%, 3% (1 year), 42%, 13% (3 years) and 61%, 19% (5 years).[32] The rate of YMDD motif mutant and BTH were low after three or more years of LMV treatment. In our data, once a BTH was experienced, FibroScan values were less likely to show improvement, which may indicate the emergence of HCC. Development of HBV resistance to LMV is typically indicated by an increase in HBV DNA followed by an increase in serum ALT level. With the advent of ETV, the frequency of viral breakthrough has been dramatically reduced;[33] however, the viral kinetics must be carefully determined.

In an analysis of the relationship between FibroScan values and the development of HCC, higher FibroScan values were shown to be associated with the development of HCC in a large prospective study of patients with chronic liver disease.[34] FibroScan values of over 10 kPa were associated with a significantly increased risk of subsequent HCC development and mortality for both chronic hepatitis B and C, irrespective of virological response owing to antiviral treatment.[35,36] Foucher *et al.*[34] suggested that the cutoff value established with a negative predictive value of greater than 90% was 53.7 kPa for HCC in patients with chronic liver diseases. Masuzaki *et al.*[35] reported that patients with chronic hepatitis C with higher FibroScan values had a significantly higher risk of HCC, with a hazard risk of 16.7 with 10.1–15 kPa, 20.9 with 15.1–20 kPa, 25.6 with 20.1–25 kPa and 45.5 with over 25 kPa, as compared to under 10 kPa. In the present study, 6 (13.3%) of 45 patients developed HCC. For those who underwent NA treatment and achieved virological response; however, the FibroScan values of most (66.7%) were consistently over 10 kPa during the follow-up period. Therefore, patients with a high FibroScan value need careful attention to prevent the development of HCC, even after achieving virological response by NA treatment.

Some limitations of FibroScan must be taken into account, such as whether or not the results are reliable. FibroScan values may be influenced by ALT flares, with a risk of overestimating liver stiffness because ALT flares reflect liver cell inflammation, edema and swelling.[36] In fact, FibroScan values of our patients transiently increased with the onset of BTH, thus FibroScan was done only after ALT level had returned to normal owing to the addition of ADV. Although the machine provides no feedback when FibroScan measurement is unsuccessful, Castéra *et al.*[37] recommended that successful measurements be validated by use of the following criteria: success rate of under 60%, IQR/M of more than 30% of median values, obesity, particularly BMI greater than 30 kg/m$^2$ and limited operator experience (fewer than 500 examinations) are the main determinants of unreliable FibroScan measurement. Another limitation is the small size of the study population; therefore, the clinical correlations with BTH or HCC development cannot be made appropriately.

In conclusion, transient elastography is a useful tool for the evaluation of liver stiffness and follow-up assessment of NA treatment of patients with chronic HBV infection. Liver stiffness, measured by transient elastog-

raphy, of chronic hepatitis B patients treated with NA showed a rapid decline in the first 3 years followed by a more steady transition from years three to five irrespective of the long term virological effect.

## REFERENCES


1 Liaw YF, Chu CM. Hepatitis B virus infection. *Lancet* 2009; **373**: 582–92.
2 Dienstag JL. Hepatitis B virus infection. *N Engl J Med* 2008; **359**: 1486–500.
3 Dienstag JL, Schiff ER, Wright TL *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256–63.
4 Nevens F, Main J, Honkoop P *et al.* Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. *Gastroenterology* 1997; **113**: 1258–63.
5 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61–8.
6 Yao FY, Bass NM. Lamivudine treatment in patients with severely decompensated cirrhosis due to replicating hepatitis B infection. *J Hepatol* 2000; **33**: 301–7.
7 Furusyo N, Takeoka H, Toyoda K *et al.* Long-term lamivudine treatment for chronic hepatitis B in Japanese patients: a project of Kyushu University Liver Disease Study. *World J Gastroenterol* 2006; **12**: 561–7.
8 Lai CL, Dienstag J, Schiff E *et al.* Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; **36**: 687–96.
9 Allen MI, Deslauriers M, Andrews CW *et al.* Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* 1998; **27**: 1670–7.
10 Manning DS, Afdhal NH. Diagnosis and quantitation of fibrosis. *Gastroenterology* 2008; **134**: 1670–81.
11 Sandrin L, Tanter M, Gennisson JL, Catheline S, Fink M. Shear elasticity probe for soft tissues with 1-D transient elastography. *IEEE Trans Ultrason Ferroelectr Freq Control* 2002; **49**: 436–46.
12 Wong VW, Vergniol J, Wong GL *et al.* Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* 2010; **51**: 454–62.
13 Corpechot C, El Naggar A, Poujol-Robert A *et al.* Assessment of biliary fibrosis by transient elastography in patients with PBC and PSC. *Hepatology* 2006; **43**: 1118–24.
14 Ziol M, Handra-Luca A, Kettaneh A *et al.* Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48–54.
15 Ogawa E, Furusyo N, Toyoda K *et al.* Transient elastography for patients with chronic hepatitis B and C virus infection: non-invasive, quantitative assessment of liver fibrosis. *Hepatol Res* 2007; **37**: 1002–10.
16 Ogawa E, Furusyo N, Toyoda K, Takeoka H, Maeda S, Hayashi J. The longitudinal quantitative assessment by transient elastography of chronic hepatitis C patients treated with pegylated interferon alpha-2b and ribavirin. *Antiviral Res* 2009; **83**: 127–34.
17 Chan HL, Tsang SW, Liew CT *et al.* Viral genotype and hepatitis B virus DNA levels are correlated with histological liver damage in HBeAg-negative chronic hepatitis B virus infection. *Am J Gastroenterol* 2002; **97**: 406–12.
18 Lok AS, Zoulim F, Locarnini S *et al.* Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007; **46**: 254–65.
19 Whalley SA, Brown D, Teo CG, Dusheiko GM, Saunders NA. Monitoring the emergence of hepatitis B virus polymerase gene variants during lamivudine therapy using the LightCycler. *J Clin Microbiol* 2001; **39**: 1456–9.
20 Murata M, Furusyo N, Unno M *et al.* Long-term effects of lamivudine treatment in Japanese chronic hepatitis B patients. *World J Gastroenterol* 2011; **17**: 2945–52.
21 Lucidarme D, Foucher J, Le Bail B *et al.* Factors of accuracy of transient elastography (fibroscan) for the diagnosis of liver fibrosis in chronic hepatitis C. *Hepatology* 2009; **49**: 1083–9.
22 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.
23 Enomoto M, Mori M, Ogawa T *et al.* Usefulness of transient elastography for assessment of liver fibrosis in chronic hepatitis B: regression of liver stiffness during entecavir therapy. *Hepatol Res* 2010; **40**: 853–61.
24 Wong GL, Wong VW, Choi PC *et al.* On-treatment monitoring of liver fibrosis with transient elastography in chronic hepatitis B patients. *Antivir Ther* 2011; **16**: 165–72.
25 Suzuki Y, Arase Y, Ikeda K *et al.* Histological improvements after a three-year lamivudine therapy in patients with chronic hepatitis B in whom YMDD mutants did not or did develop. *Intervirology* 2003; **46**: 164–70.
26 Liaw YF, Sung JJ, Chow WC *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351**: 1521–31.
27 Chen CJ, Yang HI, Su J *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65–73.
28 Kao JH. Hepatitis B viral genotypes: clinical relevance and molecular characteristics. *J Gastroenterol Hepatol* 2002; **17**: 643–50.
29 Scotto G, Fazio V. Hepatitis B genotypes and response to adefovir treatment in patients with HBV chronic infection. *J Infect* 2010; **60**: 399–401.
30 Furusyo N, Nakashima H, Kashiwagi K *et al.* Clinical outcomes of hepatitis B virus (HBV) genotypes B and C in

Japanese patients with chronic HBV infection. *Am J Trop Med Hyg* 2002; **67**: 151–7.
31 Sugiyama M, Tanaka Y, Kato T *et al.* Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* 2006; **44**: 915–24.
32 Kobayashi M, Suzuki F, Akuta N *et al.* Correlation of YMDD mutation and breakthrough hepatitis with hepatitis B virus DNA and serum ALT during lamivudine treatment. *Hepatol Res* 2010; **40**: 125–34.
33 Gish RG, Lok AS, Chang TT *et al.* Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. *Gastroenterology* 2007; **133**: 1437–44.
34 Foucher J, Chanteloup E, Vergniol J *et al.* Diagnosis of cirrhosis by transient elastography (FibroScan): a prospective study. *Gut* 2006; **55**: 403–8.
35 Masuzaki R, Tateishi R, Yoshida H *et al.* Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. *Hepatology* 2009; **49**: 1954–61.
36 Arena U, Vizzutti F, Corti G *et al.* Acute viral hepatitis increases liver stiffness values measured by transient elastography. *Hepatology* 2008; **47**: 380–4.
37 Castéra L, Foucher J, Bernard PH *et al.* Pitfalls of liver stiffness measurement: a 5-year prospective study of 13 369 examinations. *Hepatology* 2010; **51**: 828–35.

Clinical & Experimental Immunology
The Journal of Translational Immunology
British Society for immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2010.04246.x

# Prolonged recurrence-free survival following OK432-stimulated dendritic cell transfer into hepatocellular carcinoma during transarterial embolization

Y. Nakamoto,* E. Mizukoshi,* M. Kitahara,* F. Arihara,* Y. Sakai,* K. Kakinoki,* Y. Fujita,* Y. Marukawa,* K. Arai,* T. Yamashita,* N. Mukaida,† K. Matsushima,‡ O. Matsui§ and S. Kaneko*

**Disease Control and Homeostasis, Graduate School of Medicine, †Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, §Department of Radiology, Graduate School of Medicine, Kanazawa University, Kanazawa, and ‡Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*

Accepted for publication 19 July 2010
Correspondences: S. Kaneko, Disease Control and Homeostasis, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan.
E-mail: skaneko@m-kanazawa.jp

## Summary

Despite curative locoregional treatments for hepatocellular carcinoma (HCC), tumour recurrence rates remain high. The current study was designed to assess the safety and bioactivity of infusion of dendritic cells (DCs) stimulated with OK432, a streptococcus-derived anti-cancer immunotherapeutic agent, into tumour tissues following transcatheter hepatic arterial embolization (TAE) treatment in patients with HCC. DCs were derived from peripheral blood monocytes of patients with hepatitis C virus-related cirrhosis and HCC in the presence of interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor and stimulated with 0·1 KE/ml OK432 for 2 days. Thirteen patients were administered with $5 \times 10^6$ of DCs through arterial catheter during the procedures of TAE treatment on day 7. The immunomodulatory effects and clinical responses were evaluated in comparison with a group of 22 historical controls treated with TAE but without DC transfer. OK432 stimulation of immature DCs promoted their maturation towards cells with activated phenotypes, high expression of a homing receptor, fairly well-preserved phagocytic capacity, greatly enhanced cytokine production and effective tumoricidal activity. Administration of OK432-stimulated DCs to patients was found to be feasible and safe. Kaplan–Meier analysis revealed prolonged recurrence-free survival of patients treated in this manner compared with the historical controls ($P = 0{\cdot}046$, log-rank test). The bioactivity of the transferred DCs was reflected in higher serum concentrations of the cytokines IL-9, IL-15 and tumour necrosis factor-α and the chemokines CCL4 and CCL11. Collectively, this study suggests that a DC-based, active immunotherapeutic strategy in combination with locoregional treatments exerts beneficial anti-tumour effects against liver cancer.

**Keywords:** dendritic cells, hepatocellular carcinoma, immunotherapy, recurrence-free survival, transcatheter hepatic arterial embolization

## Introduction

Many locoregional therapeutic approaches including surgical resection, radiofrequency ablation (RFA) and transcatheter hepatic arterial embolization (TAE) have been taken in the search for curative treatments of hepatocellular carcinoma (HCC). Despite these efforts, tumour recurrence rates remain high [1,2], probably because active hepatitis and cirrhosis in the surrounding non-tumour liver tissues causes *de novo* development of HCC [3,4]. One strategy to reduce tumour recurrence is to enhance anti-tumour immune responses that may induce sufficient inhibitory effects to prevent tumour cell growth and survival [5,6]. Dendritic cells (DCs) are the most potent type of antigen-presenting cells in the human body, and are involved in the regulation of both innate and adaptive immune responses [7]. DC-based immunotherapies are believed to contribute to the eradication of residual and recurrent tumour cells.

To enhance tumour antigen presentation to T lymphocytes, DCs have been transferred with major histocompatibility complex (MHC) class I and class II genes [8] and co-stimulatory molecules, e.g. CD40, CD80 and CD86 [9,10], and loaded with tumour-associated antigens, including tumour lysates, peptides and RNA transfection [11]. To induce natural killer (NK) and natural killer T (NK T) cell activation, DCs have been stimulated and modified to

**Table 1.** Patient characteristics.

| Patient no. | Gender | Age (years) | HLA | TNM stages | No. of tumours | Largest tumour (mm) | Child–Pugh | KPS | Post-TAE Rx |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 60 | A11 A33 | III | 5 | 35 | B | 100 | RFA |
| 2 | M | 57 | A11 A24 | III | 1 | 21 | B | 100 | RFA |
| 3 | M | 57 | A11 A31 | III | 2 | 39 | B | 100 | RFA |
| 4 | M | 77 | A2 A24 | III | 2 | 35 | A | 100 | RFA |
| 5 | F | 83 | A11 A24 | III | 3 | 29 | B | 100 | RFA |
| 6 | F | 74 | A2 A24 | II | 1 | 35 | A | 100 | RFA |
| 7 | F | 72 | A24 A33 | III | 3 | 41 | B | 100 | RFA |
| 8 | F | 65 | A2 A11 | II | 4 | 12 | B | 100 | RFA |
| 9 | M | 71 | A2 A11 | II | 4 | 16 | A | 100 | RFA |
| 10 | M | 79 | A11 A24 | III | 2 | 40 | A | 100 | RFA |
| 11 | M | 71 | A2 A24 | II | 1 | 28 | A | 100 | RFA |
| 12 | M | 56 | A2 A26 | III | 2 | 25 | B | 100 | RFA |
| 13 | M | 64 | A2 A33 | III | 2 | 37 | B | 100 | RFA |

M, male; F, female; TNM, tumour–node–metastasis; Child–Pugh, Child–Pugh classification; KPS, Karnofsky performance scores; TAE, transcatheter arterial embolization; Rx, treatment; HCC, hepatocellular carcinoma; HLA, human leucocyte antigen; RFA, percutaneous radiofrequency ablation.

produce larger amounts of cytokines, e.g. interleukin (IL)-12, IL-18 and type I interferons (IFNs)[10,12]. Furthermore, DC migration into secondary lymphoid organs could be induced by expression of chemokine genes, e.g. C-C chemokine receptor-7 (CCR7) [13], and by maturation using inflammatory cytokines [14], matrix metalloproteinases and Toll-like receptor (TLR) ligands [15].

DCs stimulated with OK432, a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*, were suggested recently to produce large amounts of T helper type 1 (Th1) cytokines, including IL-12 and IFN-$\gamma$ and enhance cytotoxic T lymphocyte activity compared to a standard mixture of cytokines [tumour necrosis factor-$\alpha$ (TNF-$\alpha$), IL-1$\beta$, IL-6 and prostaglandin $E_2$ ($PGE_2$)] [16]. Furthermore, because OK432 modulates DC maturation through TLR-4 and the $\beta_2$ integrin system [16,17] and TLR-4-stimulated DCs can abrogate the activity of regulatory T cells [18], OK432-stimulated DCs may contribute to the induction of anti-tumour immune responses partly by reducing the activity of suppressor cells. Recently, in addition to the orchestration of immune responses, OK432-activated DCs have themselves been shown to mediate strong, specific cytotoxicity towards tumour cells via CD40/CD40 ligand interactions [19].

We have reported recently that combination therapy using TAE together with immature DC infusion is safe for patients with cirrhosis and HCC [20]. DCs were infused precisely into tumour tissues and contributed to the recruitment and activation of immune cells *in situ*. However, this approach by itself yielded limited anti-tumour effects due probably to insufficient stimulation of immature DCs (the preparation of which seems closely related to therapeutic outcome [21,22]). The current study was designed to assess the safety and bioactivity of OK432-stimulated DC infusion into tumour tissues following TAE treatment in patients with cirrhosis and HCC. In addition to documenting the safety of this approach, we found that patients treated with OK432-stimulated DCs displayed unique cytokine and chemokine profiles and, most importantly, experienced prolonged recurrence-free survival.

## Patients and methods

### Patients

Inclusion criteria were a radiological diagnosis of primary HCC by computed tomography (CT) angiography, hepatitis C virus (HCV)-related HCC, a Karnofsky score of $\geq$ 70%, an age of $\geq$ 20 years, informed consent and the following normal baseline haematological parameters (within 1 week before DC administration): haemoglobin $\geq$ 8·5 g/dl; white cell count $\geq$ 2000/$\mu$l; platelet count $\geq$ 50 000/$\mu$l; creatinine $<$ 1·5 mg/dl and liver damage A or B [23].

Exclusion criteria included severe cardiac, renal, pulmonary, haematological or other systemic disease associated with a discontinuation risk; human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy or radiation therapy within 4 weeks; immunological disorders including splenectomy and radiation to the spleen; corticosteroid or anti-histamine therapy; current lactation; pregnancy; history of organ transplantation; or difficulty in follow-up.

Thirteen patients (four women and nine men) presenting at Kanazawa University Hospital between March 2004 and June 2006 were enrolled into the study, with an age range from 56 to 83 years (Table 1). Patients with verified radiological diagnoses of HCC stage II or more were eligible and enrolled in this study. In addition, a group of 22 historical controls (nine women and 13 men) treated with TAE without DC administration between July 2000 and September 2007 was included in this study. All patients received RFA therapy to increase the locoregional effects 1 week later [24].

They underwent ultrasound, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the abdomen about 1 month after treatment and at a minimum of once every 3 months thereafter, and tumour recurrences were followed for up to 360 days. The Institutional Review Board reviewed and approved the study protocol. This study complied with ethical standards outlined in the Declaration of Helsinki. Adverse events were monitored for 1 month after the DC infusion in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

### Preparation and injection of autologous DCs

DCs were generated from blood monocyte precursors, as reported previously [25]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in six-well tissue culture dishes (Costar, Cambridge, MA, USA) at $1{\cdot}4 \times 10^7$ cells in 2 ml per well and allowed to adhere to plastic for 2 h. Adherent cells were cultured in serum-free media (GMP CellGro® DC Medium; CellGro, Manassas, VA, USA) with 50 ng/ml recombinant human IL-4 (GMP grade; CellGro®) and 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (GMP grade; CellGro®) for 5 days to generate immature DC, and matured for a further 2 days in 0·1 KE/ml OK432 (Chugai Pharmaceuticals, Tokyo, Japan) to generate OK-DC. On day 7, the cells were harvested for injection, $5 \times 10^6$ cells were suspended in 5 ml normal saline containing 1% autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ, USA) and infused through an arterial catheter following Lipiodol (iodized oil) (Lipiodol Ultrafluide, Laboratoire Guerbet, Aulnay-Sous-Bois, France) injection during selective TAE therapy. Release criteria for DCs were viability > 80%, purity > 30%, negative Gram stain and endotoxin polymerase chain reaction (PCR) and negative in process cultures from samples sent 48 h before release. All products met all release criteria, and the DCs had a typical phenotype of $CD14^-$ and human leucocyte antigen (HLA)-$DR^+$.

### Flow cytometry analysis

The DC preparation was assessed by staining with the following monoclonal antibodies for 30 min on ice: anti-lineage cocktail 1 (lin-1; CD3, CD14, CD16, CD19, CD20 and CD56)-fluorescein isothiocyanate (FITC), anti-HLA-DR-peridinin chlorophyll protein (PerCP) (L243), anti-CCR7-phycoerythrin (PE) (3D12) (BD PharMingen, San Diego, CA, USA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA, USA). Cells were analysed on a fluorescence activated cell sorter (FACS0Calibur™ flow cytometer. Data analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA, USA).

### DC phagocytosis

Immature DCs and OK432-stimulated DCs were incubated with 1 mg/ml FITC dextran (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C and the cells were washed three times in FACS buffer before cell acquisition using a FACS-Calibur™ cytometer. Control DCs (not incubated with FITC dextran) were acquired at the same time to allow background levels of fluorescence to be determined.

### Enzyme-linked immunosorbent assay (ELISA)

DCs were seeded at 200 000 cells/ml, and supernatant collected after 48 h. IL-12p40 and IFN-γ were detected using matched paired antibodies (BD Pharmingen) following standard protocols.

### Cytotoxicity assays

The ability of DCs to exert cytotoxicity was assessed in a standard $^{51}Cr$ release assay [19]. We used the HCC cell lines Hep3B and PLC/PRF/5 [American Type Culture Collection (ATCC), Manassas, VA, USA] and a lymphoblastoid cell line T2 that expresses HLA-A*0201 (ATCC) as target cells. Target cells were labelled with $^{51}Cr$. In a 96-well plate, $2{\cdot}5 \times 10^3$ target cells per well were incubated with DCs for 8 h at different effector/target (E/T) ratios in triplicate. Percentage of specific lysis was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Spontaneous release was always < 20% of the total.

### NK cell activity

NK cell cytotoxicity against K562 erythroleukemia target cells was measured by using $^{51}Cr$-release assay, according to previously published methods [26], with PBMCs obtained from the patients. All experiments were performed in triplicate. Percentage of cytotoxicity was calculated as follows: {[experimental counts per minute (cpm) – spontaneous cpm]/[total cpm – spontaneous cpm]} × 100.

### Intracellular cytokine expression

Freshly isolated PBMCs were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) at 37°C in humidified 7% $CO_2$ for 4 h. To block cytokine secretion, brefeldin A (Sigma) [27] was added to a final concentration of 10 μg/ml. After addition of stimuli, the surface staining was performed with anti-CD4-PC5 (13B8·2), anti-CD8-PerCP (SK1) and anti-CD56-PC5 (N901) (Beckman

Coulter). Subsequently, the cells were permeabilized, stained for intracellular IFN-γ and IL-4 using the FastImmune™ system (BD Pharmingen), resuspended in phosphate-buffered saline (PBS) containing 1% paraformaldehyde (PFA), and analysed on a flow cytometer (≈ 10 000 gated events acquired per sample).

### IFN-γ enzyme-linked immunospot (ELISPOT) assay

ELISPOT assays were performed as described previously with the following modifications [28–30]. HLA-A24 restricted peptide epitopes, squamous cell carcinoma antigen recognized by T cells 2 $(SART2)_{899}$ (SYTRLFLIL), $SART3_{109}$ (VYDYNCHVDL), multi-drug resistance protein 3 $(MRP3)_{765}$ (VYSDADIFL), $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), alpha-fetoprotein $(AFP)_{403}$ (KYIQESQAL), $AFP_{434}$ (AYTKKAPQL), $AFP_{357}$ (EYSRRHPQL), human telomerase reverse transcriptase $(hTERT)_{167}$ (AYQVCGPPL) (unpublished), $hTERT_{461}$ (VYGFVRACL) and $hTERT_{324}$ (VYAETKHFL) were used in this study. Negative controls consisted of an HIV envelope-derived peptide ($HIVenv_{584}$). Positive controls consisted of 10 ng/ml PMA (Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$). The coloured spots were counted with a KS ELISPOT Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of antigen from the number of spots in its presence. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen.

### Cytokine and chemokine profiling

Serum cytokine and chemokine levels were measured using the Bioplex assay (Bio-Rad, Hercules, CA, USA). Briefly, frozen serum samples were thawed at room temperature, diluted 1:4 in sample diluents, and 50 µl aliquots of diluted sample were added in duplicate to the wells of a 96-well microtitre plate containing the coated beads for a validated panel of 27 human cytokines and chemokines (cytokine 27-plex antibody bead kit) according to the manufacturer's instructions. These included IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, G-CSF, GM-CSF, IFN-γ, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, MIP-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated upon activation normal T cell-expressed and secreted (RANTES), TNF-α and vascular endothelial growth factor (VEGF). Eight standards (ranging from 2 to 32 000 pg/ml) were used to generate calibration curves for each cytokine. Data acquisition and analysis were performed using Bio-Plex Manager software version 4.1.1.

### Arginase activity

Serum samples were tested for arginase activity by conversion of L-arginine to L-ornithine [31] using a kit supplied by the manufacturer (BioAssay Systems, Hayward, CA, USA). Briefly, sera were treated with a membrane filter (Millipore, Billerica, MA, USA) to remove urea, combined with the sample buffer in wells of a 96-well plate, and incubated at 37°C for 2 h. Subsequently, the urea reagent was added to stop the arginase reaction. The colour produced was read at 520 nm using a microtitre plate reader.

### Statistical analysis

Results are expressed as means ± standard deviation (s.d.). Differences between groups were analysed for statistical significance by the Mann–Whitney *U*-test. Qualitative variables were compared by means of Fisher's exact test. The estimated probability of tumour recurrence-free survival was determined using the Kaplan–Meier method. The Mantel–Cox log-rank test was used to compare curves between groups. Any *P*-values less than 0·05 were considered statistically significant. All statistical tests were two-sided.

## Results

### Preparation of OK432-stimulated DCs

Adherent cells isolated from PBMCs of patients with cirrhosis and HCC (Table 1) were differentiated into DCs in the presence of IL-4 and GM-CSF. The cells were stimulated with 0·1 KE/ml OK432 for 3 days; 54·6 ± 9·5% (mean ± s.d.; $n = 13$) of OK432-stimulated cells showed high levels of MHC class II (HLA-DR) and the absence of lineage markers including CD3, CD14, CD16, CD19, CD20 and CD56, in which 30·9 ± 14·2% were CD11c-positive (myeloid DC subset) and 14·8 ± 11·2 were CD123-positive (plasmacytoid DC subset), consistent with our previous observations [20]. As reported [32,33], greater proportions of the cells developed high levels of expression of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) and an activation marker (CD83) compared to DCs prepared without OK432 stimulation (Fig. 1a). Furthermore, the chemokine receptor CCR7 which leads to homing to lymph nodes [13,34] was also induced following OK432 stimulation.

To evaluate the endocytic and phagocytic ability of the OK432-stimulated cells, uptake of FITC-dextran was quantitated by flow cytometry (Fig. 1b). The cells showed lower levels of uptake due to maturation compared to DCs prepared without OK432 stimulation, while the OK432-stimulated cells derived from HCC patients preserved a moderate uptake capacity. As expected, the OK432-stimulated cells produced large amounts of cytokines IL-12 and IFN-γ (Fig. 1c). In addition, they displayed high cyto-

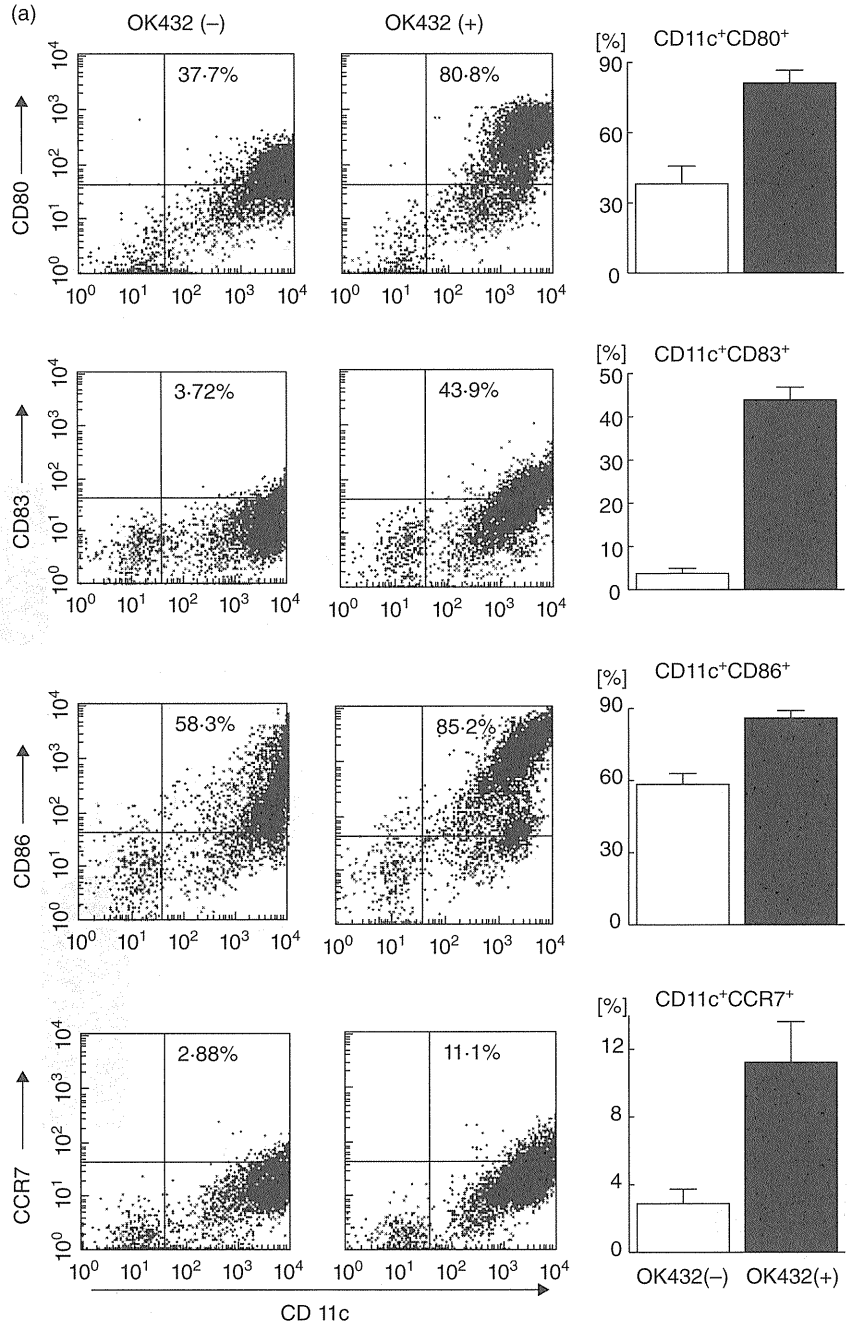


**Fig. 1.** Effects of OK432 stimulation on the properties of dendritic cells (DCs) generated from blood monocyte precursors in patients with cirrhosis and hepatocellular carcinoma (HCC) ($n = 13$). (a) Lineage cocktail 1 (lin $1^-$) human leucocyte antigen D-related (HLA-$DR^-$) subsets with [OK432(+)] and without [OK432(−)] stimulation were analysed for surface expression of CD80, CD83, CD86 and CCR7. Dot plots of a representative case are shown in the left-hand panel. Mean percentages [±standard deviation (s.d.)] of positive cells are indicated in the right-hand panel. OK432 stimulation resulted in the expression of high levels of CD80, CD83, CD86 and CCR7 in the lin $1^-$human leucocyte antigen D-related (HLA-$DR^-$) DC subset. (b) DC subsets with and without OK432 stimulation were incubated with fluorescein isothiocyanate (FITC) dextran for 30 min and the uptake was determined by flow cytometry. A representative analysis is shown in the upper panel. Mean fluorescence intensities (MFIs) (±s.d.) of the positive cells are indicated in the lower panel. OK432-stimulated cells showed lower levels of uptake due to maturation. (c) DC supernatants were harvested and the concentrations of interleukin (IL)-12 and interferon (IFN)-γ measured by enzyme-linked immunosorbent assay (ELISA). OK432-stimulated cells produced large amounts of the cytokines. The data indicate means ± s.d. of the groups with and without the stimulation. All comparisons in (a–c) [OK432(+) *versus* OK432(−)] were statistically significant by the Mann-Whitney *U*-test ($P < 0{\cdot}005$). (d) Tumoricidal activity of DCs assessed by incubation with $^{51}$Cr-labelled Hep3B, PLC/PRF/5 and T2 targets for 8 h at the indicated effector/target (E/T) cell ratios. OK432-stimulated cells displayed high cytotoxic activity against the target cells. The results are representative of the cases studied.

toxic activity against HCC cell lines (Hep3B and PLC/PRF/5) and a lymphoblastoid cell line (T2) although DCs without OK432 stimulation lysed none of the target cells to any great degree (Fig. 1d). Taken together, these results demonstrate that OK432 stimulation of IL-4 and GM-CSF-induced immature DCs derived from HCC patients promoted their maturation towards cells with activated phenotypes, high expression of a homing receptor, fairly well-preserved phagocytic capacity, greatly enhanced cytokine production and effective tumoricidal activity, consistent with previous observations [16,19].

## Safety of OK432-stimulated DC administration

Prior to the administration of OK432-stimulated DCs to patients, the cells were confirmed to be safe in athymic nude mice to which 100-fold cell numbers/weight were injected subcutaneously (data not shown). Subsequently, OK432-stimulated DC administration was performed during TAE therapy in humans, in which DCs were mixed together with absorbable gelatin sponge (Gelfoam) and infused through an arterial catheter following iodized oil (Lipiodol) injection, as reported previously [20]. Adverse events were

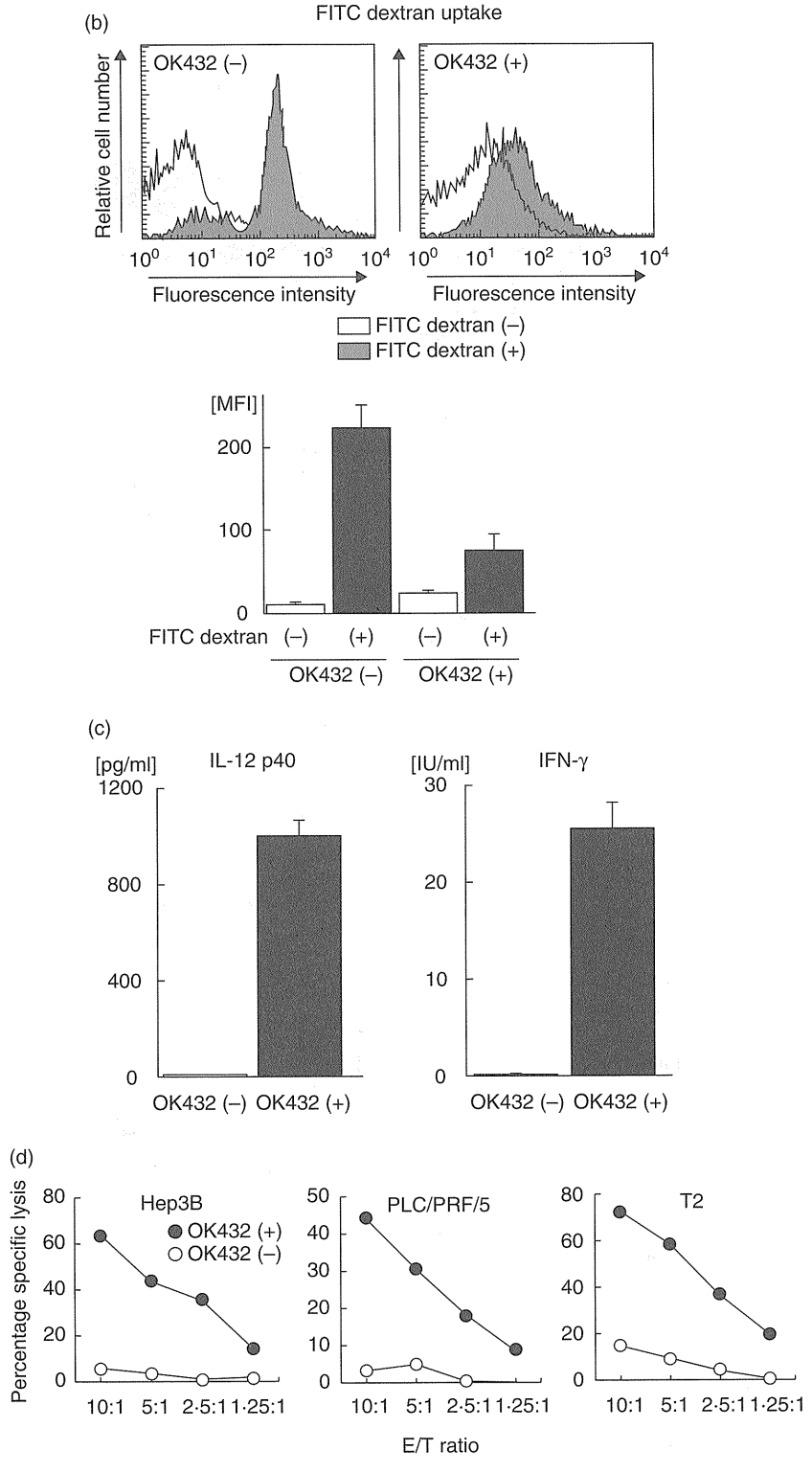


**Fig. 1.** *Continued*

monitored clinically and biochemically after DC infusion (Table 2). A larger proportion (12 of 13) of the patients were complicated with high fever compared to those treated previously with immature DCs (five of 10) [20], due probably to the proinflammatory responses induced by OK432-stimulated DCs. However, there were no grades III or IV National Cancer Institute Common Toxicity Criteria adverse events, including vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorders, bleeding, hepatic abscess or autoimmune diseases associated with DC infusion and TAE in this study. There was also no clinical or serological evidence of hepatic failure or autoimmune