Table 1
Baseline characteristics of study patients ($n = 144$).

Age (years)	58.3 ± 8.9
Sex (female/male)	71 (49.3)/73 (50.7)
History of interferon therapy (naive/retreatment)	109 (75.7)/35 (24.3)
Body weight (kg)	58.7 ± 10.1
Alanine aminotransferase (IU/L)	62.1 ± 58.2
Aspartate aminotransferase (IU/L)	52.7 ± 40.4
Gamma-glutamyl transpeptidase (IU)	52.6 ± 58.3
Alkaline phosphatase (IU/L)	260.6 ± 83.9
Albumin (g/dL)	4.15 ± 0.35
Total bilirubin (mg/dL)	0.68 ± 0.30
White blood cell count (/μL)	5130 ± 1327
Hemoglobin (g/dL)	14.2 ± 1.4
Platelet count (× 10^3/μL)	167 ± 51
Liver histology-activity (A0/A1/A2/A3)[a]	3 (2.3)/73 (54.9)/45 (33.8)/12 (9.0)
Liver histology-fibrosis (F0/F1/F2/F3)[a]	5 (3.8)/79 (59.4)/33 (24.8)/16 (12.0)
Pretreatment HCV RNA concentration ($\log_{10}$ IU/mL)	6.28 ± 6.16
Reduction of the peginterferon dose	40 (27.8)
Reduction of the ribavirin dose	71 (49.3)

HCV, hepatitis C virus. Percentages are shown in parentheses.
[a] Liver biopsy was not performed in 11 patients.

ing previously gone negative. This reappearance could be observed only using real-time PCR, and all patients who showed the phenomenon relapsed after the completion of the therapy.

3. Study design

Between January 2006 and March 2008, a total of 156 patients with chronic HCV genotype 1b infection underwent antiviral combination therapy with PEG-IFN and ribavirin at our institution. Among these patients, 148 had pretreatment HCV RNA concentrations >100,000 IU/mL as assayed by quantitative Amplicor Monitor assay (AMPLICOR HCV MONITOR Test, version 2.0; Roche Molecular Systems, Pleasanton, CA). No patients with HCV genotype 1a were included because this type is not found in the general Japanese population. In this study, we included the 144 of these 148 patients who agreed to store serum samples and to have them used in the study. Table 1 shows the baseline characteristics of the 144 study patients. Although 35 patients had a history of previous antiviral monotherapy with conventional IFN or combination therapy with conventional IFN and ribavirin (retreatment cases), no patients had a history of combination therapy with PEG-IFN and ribavirin. Of 133 patients who underwent a pretreatment liver biopsy, the grade of liver fibrosis according to the METAVIR score[19] was F0 in 5 patients (3.8%), F1 in 79 patients (59.4%), F2 in 33 patients (24.8%), and F3 in 16 patients (12.0%), respectively. No patients were coinfected with hepatitis B virus or human immunodeficiency virus. No patients had histories of alcohol abuse or intravenous drug use. For combination therapy with PEG-IFN and ribavirin, all patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough) daily. The dose of PEG-IFN and ribavirin were adjusted by patient body weight. Patients weighing ≤45 kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing >45 kg and ≤60 kg were given 80 μg, those weighing >60 kg and ≤75 kg were given 100 μg, those weighing >75 kg and ≤90 kg were given 120 μg, and those weighing >90 kg were given 150 μg. Patients weighing ≤60 kg were given 600 mg of ribavirin per day, those weighing >60 kg and ≤80 kg were given 800 mg of ribavirin per day, and those weighing >80 kg were given 1000 mg of ribavirin per day. Dose modification or discontinuation of PEG-IFN or ribavirin was based on the manufacturer's recommendations. During the therapy, 40 patients (27.8%) had their PEG-IFN doses reduced and 71 patients (49.3%) had their ribavirin doses reduced. No patients discontinued the therapy. SVR was defined as undetectable serum HCV RNA throughout 24 weeks after the end of therapy. Relapse was defined as positive serum HCV RNA during the period between the end of treatment and 24 weeks thereafter, despite the disappearance of serum HCV RNA by the end of treatment. As for responses during the therapy, rapid virologic response (RVR) was defined as negative serum HCV RNA at 4 weeks after the start of the therapy. Complete early virologic response (cEVR) was defined as negative serum HCV RNA at 12 weeks after the start of the therapy.[20] Slow virologic response was defined as the disappearance of serum HCV RNA between 12 and 24 weeks after the start of the therapy. Non-response was defined as failure to clear serum HCV RNA until 24 weeks after the start of the therapy (null-response or partial response).[1] End-of-treatment response (ETR) was defined as negative serum HCV RNA at the end of the therapy.[1] HCV RNA in the serum was measured by the qualitative Amplicor Monitor HCV RNA assay (AMPLICOR Hepatitis C Virus (HCV) Test, version 2.0, Roche Molecular Systems)[21] to confirm the undetectability of serum HCV RNA, when it was unquantifiable (under the detection limit) by the quantitative Amplicor Monitor assay. Patients who showed slow virologic response were recommended to elongate the treatment duration from 48 to 72 weeks according to previously published reports.[22,23]

After a patient gave consent, serum samples were obtained at the patient's regular visit to the hospital just prior to beginning treatment, and at every 4 weeks during the treatment and during the 24-week follow-up period after the treatment. Serum samples were stored at −80 °C. We measured the HCV RNA levels in these stored serum samples using a real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test, Roche Molecular Systems), and compared the results with those from the Amplicor Monitor assays. When serum HCV RNA level was low and unquantifiable, the detection of HCV RNA was tested repeatedly and the presence or absence of serum HCV RNA was confirmed.

Quantitative values are reported as mean ± SD. Between-group differences were analyzed by Chi-square test. The study protocol was approved by the institutional review board and was in compliance with the Helsinki Declaration. Written informed consent was obtained from all patients prior to the study for use of the clinical data and serum samples.

Table 2
Responses to combination therapy with peginterferon and ribavirin evaluated by Amplicor and TaqMan assay.

	Evaluation by Amplicor	Evaluation by TaqMan
Rapid virologic response	10 (6.9)	9 (6.3)
Complete early virologic response[a]	70 (48.6)	54 (37.5)
Slow virologic response	34 (23.6)	39 (27.1)
Non-response[b]	40 (27.8)	51 (35.4)
End-of-treatment response	104 (72.2)	104 (72.2)
Sustained virologic response	63 (43.8)	63 (43.8)
Relapse	41 (28.5)	41 (28.5)

Amplicor, measured by AMPLICOR HCV MONITOR Test, version 2.0; TaqMan, measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[a] Includes patients with rapid virologic response.
[b] Patients with null-response and those with partial response.

4. Results

4.1. Response of HCV RNA during treatment and final outcomes

All patients completed the therapy. Table 2 shows the responses to the therapy evaluated by the Amplicor Monitor assay and by the TaqMan PCR assay. Based on the evaluation of serum HCV RNA by the Amplicor Monitor assay during the treatment, 10 patients (6.9%) showed RVR, 70 (48.6%) showed cEVR (including the 10 with RVR), and 34 (23.6%) showed slow virologic response. The elonga-

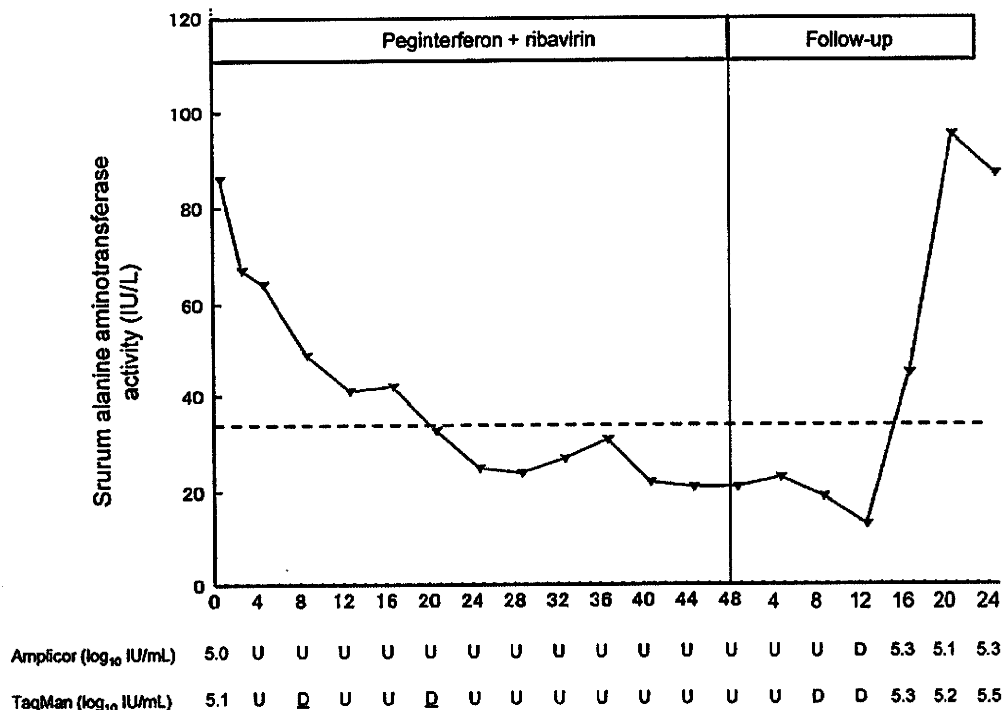

Fig. 1. Changes in serum alanine aminotransferase activity and serum HCV RNA concentration as detected by the Amplicor Monitor and TaqMan PCR assays during treatment and follow-up period (24 weeks) in a patient in whom serum HCV reappeared transiently during treatment after it had previously disappeared (patient 6). Pretreatment serum HCV RNA concentration was 5.05 log_{10} IU/mL. The Amplicor Monitor assay showed that serum HCV RNA disappeared at 4 weeks after the start of the therapy; therefore, the patient was classified as rapid virologic response. He relapsed after the end of the treatment with an increase in serum HCV RNA concentration and alanine aminotransferase activity. The TaqMan PCR assay also showed a disappearance of serum HCV RNA at 4 weeks, but this assay further showed that it reappeared transiently at 8 and 20 weeks after the start of the therapy (underline). Without the measurement of HCV RNA at 8 and 20 weeks this patient would be classified as rapid virologic response by the TaqMan PCR assay as well. If only the measurement at 20 weeks was omitted, he would be classified as complete early virologic response by the TaqMan PCR assay. "D" at Amplicor lane means that HCV RNA was under detection limit of quantitative Amplicor Monitor assay (3.70 log_{10} IU/mL) but was detected by qualitative Amplicor Monitor assay. "D" at TaqMan lane means that HCV RNA was under quantitation limit of TaqMan assay but was detected. "U" at Amplicor lane and TaqMan lane mean undetectable.

tion of the treatment duration from 48 weeks to 72 weeks was recommended for patients with slow virologic response; 17 of 34 patients (50.0%) followed the recommendation. As the final outcomes, 63 patients (43.8%) showed SVR and 41 (28.5%) relapsed. Among the 34 patients who showed the slow virologic response, the SVR rate was 5.9% (1 of 17) in the patients without the elongated treatment duration, and 41.2% (7 of 17) in the patients with it ($p = 0.0432$).

Under evaluation with the TaqMan PCR assay, 9 patients (6.3%) showed RVR, 54 (37.5%) showed cEVR (including 9 patients with RVR), and 39 (27.1%) showed slow virologic response. For 16 patients, the Amplicor Monitor assay gave the cEVR result, while the TaqMan PCR assay gave the slow virologic response result; elongation of treatment duration was not recommended for these patients because only the Amplicor Monitor assay was used during the treatment. At 24 weeks after the start of therapy, serum HCV RNA was detectable by the TaqMan PCR assay (non-response) in 11 patients for whom the Amplicor Monitor assay had given a result of slow virologic response.

4.2. Detection by TaqMan PCR assay of transient reappearance of serum HCV RNA during treatment

Using the TaqMan PCR assay, serum HCV RNA was detected again in 9 (6.3%) patients after having previously disappeared from the serum. Table 3 summarizes the data from these 9 patients. Patients 2, 5, 6, 7, 8, and 9 were categorized as cEVR by the Amplicor Monitor assay during treatment and underwent 48-week treatment. Patient 6 showed RVR during treatment (Fig. 1). Patient 1 was categorized as cEVR during treatment but strongly desired the elongation of the treatment duration and underwent 72-week treatment (Fig. 2). Patients 3 and 4 showed slow virologic response and underwent 72-week treatment. Under reanalysis of the serum samples with the TaqMan PCR assay, patients 1, 2, 6, 7, 8, and 9 remained cEVR, patient 6 remained RVR, and patients 3 and 4 remained classified as slow virologic response, when the responses were determined by the first disappearance of serum HCV RNA. However, when the reappearance was considered, patients 1, 2, 5, 6, 7, 8, and 9 actually had a slow virologic response, and serum HCV RNA remained detectable at 24 weeks after the start of the therapy (non-response) in patients 3 and 4.

Reappearance of serum HCV RNA was found at only one measurement point in 7 patients and at 2 points in the remaining 2 patients. In all case, the level of reappeared HCV RNA was low and unquantifiable despite detection. Although patients 1, 4, and 9 experienced the reduction of ribavirin dose, the reduction was not concomitant with the reappearance of serum HCV RNA. HCV RNA reappeared transiently at these points and disappeared again thereafter. In 8 patients, the reappearance was observed at the measurement point just after the first disappearance of serum HCV RNA (i.e., 4 weeks after the previous measurement). In patient 9, HCV RNA reappearance was observed at 8 weeks after the initial disappearance. In patient 6, HCV RNA first disappeared at 4 weeks after the start of the therapy but reappeared at 8 weeks. It became negative again at 12 and 16 weeks, but reappeared again at 20 weeks. In the final outcome, all 9 patients relapsed after the end of treatment regardless of treatment duration. The prevalence of relapse in patients who experienced transient reappearance of serum HCV RNA were significantly higher than those without it, by the evaluation in patients with cEVR (100% vs. 2.1%, $p < 0.0001$), in those with cEVR and slow virolgic response (100% vs. 25.0%, $p < 0.0001$), and in those with ETR (100% vs. 33.7%, $p = 0.0004$).

Table 3
Patients in whom serum HCV reappeared transiently during treatment after having previously disappeared.

	Age	Sex	History of IFN therapy	Pretreatment HCV RNA[a] ($\log_{10}$ IU/mL)	HCV RNA disappearance by Amplicor	HCV RNA disappearance by TaqMan	HCV RNA reappearance by TaqMan[b]	ALT flare during therapy	Treatment duration	Outcome
1	29	F	No	5.89	12W	12W	16W	No	72W	Relapse
2	57	F	No	6.46	12W	12W	16W	No	48W	Relapse
3	51	F	No	6.26	16W	20W	24W	No	72W	Relapse
4	65	M	No	5.66	20W	20W	24W	No	72W	Relapse
5	58	M	Yes	6.51	12W	16W	20W	No	48W	Relapse
6	65	M	Yes	5.05	4W	4W	8W and 20W	No	48W	Relapse
7	58	M	No	6.97	12W	12W	16W	No	48W	Relapse
8	61	F	No	5.99	12W	12W	16W and 20W	No	48W	Relapse
9	68	F	No	6.09	8W	12W	20W	No	48W	Relapse

M, male; F, female; W, weeks; Amplicor, measured by AMPLICOR HCV MONITOR Test, version 2.0; TaqMan, measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[a] Measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[b] HCV RNA reappeared transiently only at these measurement points and again disappeared thereafter.

5. Discussion

Measurement of HCV RNA with the real-time PCR-based TaqMan PCR assay has been reported to be superior to previous methods for the prediction of treatment outcome and the selection of a response-guided therapy regimen.[24,25] In the present study, we used the PCR-based TaqMan assay to reanalyze the changes in serum HCV RNA in patients who underwent antiviral combination therapy with PEG-IFN and ribavirin under the guidance of the Amplicor Monitor assay, in order to evaluate the usefulness of the newer technique. We found the TaqMan PCR assay to be a more sensitive detector of serum HCV RNA; it detected HCV RNA at 12 weeks after the start of the therapy in 24.3% of patients showing a cEVR result by the Amplicor Monitor assay. Umder the guidance of the TaqMan PCR assay, elongation of the treatment duration would have been recommended for these patients and their rate of SVR would presumably have increased.

More importantly, only by measurement with the TaqMan PCR assay did we observed the transient reappearance of serum HCV RNA after it had previously disappeared. Because this phenomenon was not accompanied by a flare of alanine aminotransferase and because serum HCV RNA continued to be negative by the Amplicor assay, it was missed during treatment. Breakthrough of HCV RNA during treatment is usually accompanied by an increase in serum HCV RNA concentration and a serum ALT flare, and is not transient. The phenomenon that we observed was, therefore, different from the typical breakthrough.

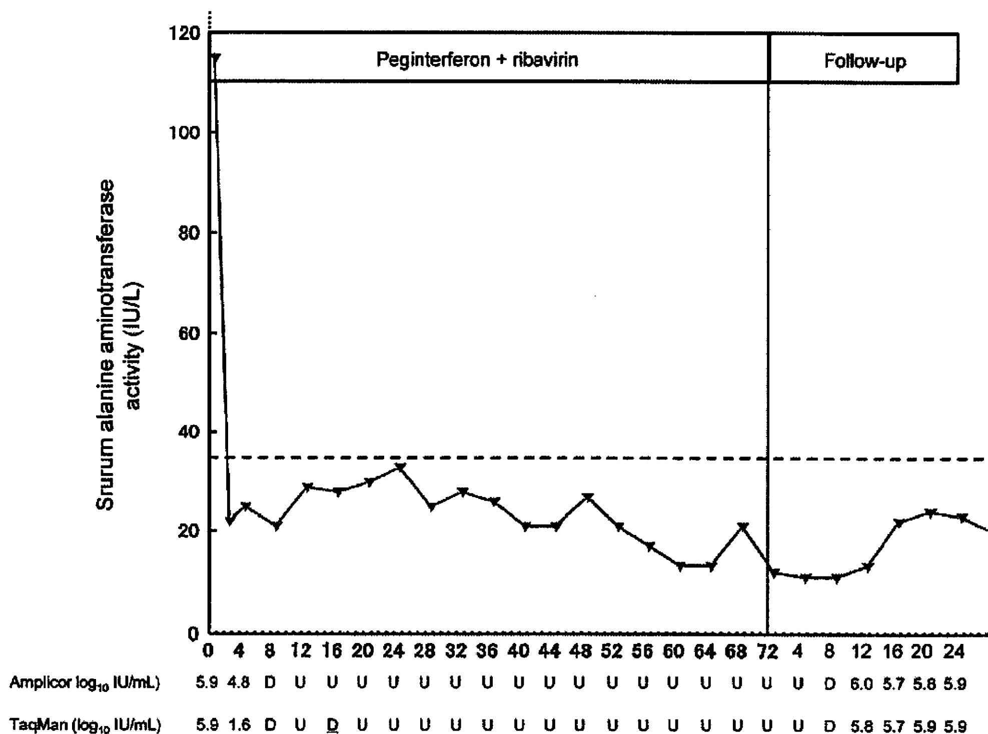

Fig. 2. Changes in serum alanine aminotransferase activity and serum HCV RNA concentration as measured by the Amplicor Monitor and TaqMan PCR assays during the treatment and follow-up period (24 weeks) in a patient in whom serum HCV reappeared transiently during treatment after having previously disappeared (patient 1). Her pretreatment serum HCV RNA concentration was 5.89 $\log_{10}$ IU/mL. The Amplicor Monitor assay showed that her serum HCV RNA disappeared at 12 weeks after the start of the therapy; therefore, she was classified as complete early virologic response. She strongly desired to elongate the treatment duration, and completed 72 weeks treatment. However, she relapsed after the end of the treatment, showing an increase in serum HCV RNA concentration. The TaqMan PCR assay also showed the disappearance of serum HCV RNA at 12 weeks, but this assay further showed that it reappeared transiently at 16 weeks after the start of the therapy (underline). "D" at Amplicor lane means that HCV RNA was under detection limit of quantitative Amplicor Monitor assay (3.70 $\log_{10}$ IU/mL) but was detected by qualitative Amplicor Monitor assay. "D" at TaqMan lane means that HCV RNA was under quantitation limit of TaqMan assay but was detected. "U" at Amplicor lane and TaqMan lane mean undetectable.

Because the phenomenon is transient, it is likely to be missed even under monitoring by the TaqMan PCR assay unless the measurement is frequently performed. In the 9 of our study patients who showed this phenomenon, reappearance of HCV RNA was found at 8, 16, or 20 weeks after the start of the therapy. Serum HCV RNA is usually measured at 4, 12, and 24 weeks after the start of the therapy[1]; therefore, any transient reappearance would be missed, unless the measurement was performed every 4 weeks. Patients 1, 2, 3, 8, and 9 would have remained cEVR and patient 6 would have remained RVR, even under measurement with the TaqMan PCR assay, if the measurement was performed only at the standard 4, 12, and 24 weeks after the start of the therapy.

In the final outcome, all these 9 patients relapsed. It is unclear why they all relapsed and no patient achieved SVR. Patients 2, 5, 6, 7, 8, and 9 would have been classified as slow virologic responders if the reappearance of HCV RNA had been detected; in these cases, the elongation of the treatment duration to 72 weeks might have resulted in SVR. In patients 3 and 4, HCV RNA was positive at 24 weeks after the start of therapy; this could explain the lack of SVR even with their 72-week treatment duration. Otherwise, a very low level of serum HCV RNA, close to the detection threshold for the TaqMan PCR assay was present throughout the treatment period, causing redetection of HCV RNA in the serum by this assay. For example, one of a few minor HCV strain that are resistant to PEG-IFN and ribavirin therapy could have been present throughout the treatment period. Further improvement of the sensitivity of the detection of serum HCV RNA will explain the results.

In conclusion, using the TaqMan PCR assay we observed a transient reappearance of serum HCV RNA after it had previously disappeared in patients with HCV genotype 1b undergoing antiviral combination therapy with PEG-IFN and ribavirin. This phenomenon is likely to be missed without frequent measurements of serum HCV RNA by sensitive detection method, and it may indicate a high likelihood of relapse after treatment even if the treatment duration is elongated. The possibility of this phenomenon should be considered during treatment in order to select the appropriate response-guided therapy. In addition, large-scale prospective studies will be needed to clarify the biological significance and clinical impact of this phenomenon.

Conflict of interest

There is no conflict of interest and there is no grant support and other assistance on this study.

References

1. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. *Hepatology* 2009;**49**:1335–74.
2. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;**345**:975–82.
3. Hadziyannis SJ, Sette Jr H, 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.
4. Shiffman ML, Di Bisceglie AM, Lindsay KL, Morishima C, Wright EC, Everson GT, et al. Peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C who have failed prior treatment. *Gastroenterology* 2004;**126**:1015–23.
5. Zeuzem S, Diago M, Gane E, Reddy KR, Pockros P, Prati D, et al. Peginterferon alfa-2a (40 kilodaltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. *Gastroenterology* 2004;**127**:1724–32.
6. Bruno S, Camma C, Di Marco V, Rumi M, Vinci M, Camozzi M, et al. Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: a randomized controlled trial. *J Hepatol* 2004;**41**:474–81.
7. Brandao C, Barone A, Carrilho F, Silva A, Patelli M, Caramori C, et al. The results of a randomized trial looking at 24 weeks vs. 48 weeks of treatment with peginterferon α-2a (40 kDa) and ribavirin combination therapy in patients with chronic hepatitis C genotype 1. *J Viral Hepatol* 2006;**13**:552–9.
8. Yu ML, Dai CY, Lin ZY, Lee LP, Hou NJ, Hsieh MY, et al. A randomized trial of 24- vs. 48-week course of PEG interferon α-2b plus ribavirin for genotype-1b-infected chronic hepatitis C patients: a pilot study in Taiwan. *Liver Int* 2006;**26**:73–81.
9. Zeuzem S, Herrmann E, Lee JH, Fricke J, Neumann AU, Modi M, et al. Viral kinetics in patients with chronic hepatitis C treated with standard or peginterferon alpha2a. *Gastroenterology* 2001;**120**:1438–47.
10. Buti M, Sanchez-Avila F, Lurie Y, Stalgis C, Valdes A, Martell M, et al. Viral kinetics in genotype 1 chronic hepatitis C patients during therapy with 2 different doses of peginterferon alfa-2b plus ribavirin. *Hepatology* 2002;**35**:930–6.
11. Berg T, Sarrazin C, Herrmann E, Hinrichsen H, Gerlach T, Zachoval R, et al. Prediction of treatment outcome in patients with chronic hepatitis C: significance of baseline parameters and viral dynamics during therapy. *Hepatology* 2003;**37**:600–9.
12. Lee SS, Ferenci P. Optimizing outcomes in patients with hepatitis C virus genotype 1 or 4. *Antiviral Ther* 2008;**13**(Suppl. 1):9–16.
13. Marcellin P, Rizzetto M. Response-guided therapy: optimizing treatment now and in the future. *Antiviral Ther* 2008;**13**(Suppl. 1):1–2.
14. Lunel F, Cresta P, Vitour D, Payan C, Dumont B, Frangeul L, et al. Comparative evaluation of hepatitis C virus RNA quantitation by branched DNA, NASBA, and Monitor assays. *Hepatology* 1999;**29**:528–35.
15. Otagiri H, Fukuda Y, Nakano I, Katano Y, Toyoda H, Yokozaki S, et al. Evaluation of a new assay for hepatitis C virus genotyping and viral load determination in patients with chronic hepatitis C. *J Virol Methods* 2002;**103**:137–43.
16. Colucci G, Ferguson J, Harkleroad C, Lee S, Romo D, Soviero S, et al. Improved COBAS TaqMan hepatitis C virus test (version 2.0) for use with the High Pure system: enhanced genotype inclusivity and performance characteristics in a multisite study. *J Clin Microbiol* 2007;**45**:3595–600.
17. Pittaluga F, Allice T, Abate ML, Ciancio A, Cerutti F, Varetto S, et al. Clinical evaluation of the COBAS Ampliprep/COBAS TaqMan for HCV RNA quantitation in comparison with the branched-DNA assay. *J Med Virol* 2008;**80**:254–60.
18. Sarrazin C, Dragan A, Gärtner BC, Forman MS, Traver S, Zeuzem S, et al. Evaluation of an automated highly sensitive real-time PCR-based assay (COBAS Ampliprep/COBAS TaqMan) for quantification of HCV RNA. *J Clin Virol* 2008;**43**:162–8.
19. The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. *Hepatology* 1994;**20**:15–20.
20. Marcellin P, Jensen DM, Hadziyannis SJ, Ferenci P. Differentiation of early virologic response (EVR) into RVR, complete EVR (cEVR) and partial EVR (pEVR) allows for a more precise prediction of SVR in HCV genotype 1 patients treated with peginterferon alfa-2a (40 kDa) (PEGASYS) and ribavirin (COPEGUS). *Hepatology* 2007;**46**(Suppl. 1):818A–9A.
21. Nolte FS, Fried MW, Shiffman ML, Ferreira-Gonzalez A, Garrett CT, Schiff ER, et al. Prospective multicenter clinical evaluation of AMPLICOR and COBAS AMPLICOR hepatitis C virus test. *J Clin Microbiol* 2001;**39**:4005–12.
22. Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006;**130**:1086–97.
23. Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis C genotype 1-infected slow responders. *Hepatology* 2007;**46**:1688–94.
24. Berg T, Weich V, Teuber G, Klinker H, Moller B, Rasenack J, et al. Individualized treatment strategy according to early viral kinetics in hepatitis C virus type 1-infected patients. *Hepatology* 2009;**50**:369–77.
25. 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.

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Incidence of Hepatocellular Carcinoma in Patients With Chronic Hepatitis B Virus Infection Who Have Normal Alanine Aminotransferase Values

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The importance of alanine aminotransferase (ALT) levels in the progression of hepatitis B virus (HBV) infection remains a subject of debate. This study sought to identify independent risk factors involved in development of hepatocellular carcinoma (HCC), particularly in patients with chronic HBV infection who have normal ALT values. Data from 381 consecutive hepatitis B patients were analyzed with average ALT integration values $\leq$40 IU/L and follow-up periods of >3 years. Integration values were calculated from biochemical tests, and serological markers associated with the cumulative incidence of HCC were analyzed. HCC developed in 17 of the 381 patients (4.5%) during the follow-up period. Male sex (hazard ratio, 6.011 [95% confidence interval: 1.353–26.710], $P=0.018$), high HBV-DNA levels ($\geq$5.0 log copies/ml; 5.125 [1.880–13.973], $P=0.001$), low platelet counts (<15.0×10^4/mm^3; 4.803 [1.690–13.647], $P=0.003$), and low total cholesterol levels (<130 mg/dl; 5.983 [1.558–22.979], $P=0.009$) were significantly associated with greater incidence of HCC development. High HBV-DNA levels and low platelet counts are associated with the development of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention of HCC in patients with low platelet counts, particularly in patients whose ALT values fall within the current normal range. ***J. Med. Virol. 82:539–545, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus (HBV); HBV-DNA; normal alanine aminotransferase; platelet counts; hepatocellular carcinoma

INTRODUCTION

Worldwide, an estimated 350 million individuals are infected chronically with hepatitis B virus (HBV), and 1 million die each year from HBV-related liver disease [EASL Jury, 2003]. Chronic HBV infection is a major risk factor for the development of hepatocellular carcinoma (HCC) [Beasley, 1988; EASL Jury, 2003]. Patients who test positive for the hepatitis B surface antigen (HBsAg) have a 70-fold greater risk of developing HCC compared with HBsAg-negative patients [Szmuness, 1978; Beasley et al., 1981]. HBV infection is endemic in Southeast Asia, China, Taiwan, Korea, and sub-Saharan Africa, where up to 85–95% of patients with HCC are HBsAg-positive [Rustgi, 1987]. HCC is the third and fifth leading cause of death from malignant neoplasms in Japanese men and women, respectively, and the death rate from HCC has increased markedly in Japan since 1975 [Kiyosawa et al., 2004]. Hepatitis C virus (HCV)-related HCC accounts for 75% of all cases of HCC in Japan, while HBV-related HCC accounts for 15% of such cases [Kiyosawa et al., 2004].

Although an increasing body of epidemiological and molecular evidence suggests that HBV is associated with the development of HCC, the exact role of HBV in carcinogenesis is unclear [Ikeda et al., 2005; Wong et al., 2006]. HBV elicits a chronic necroinflammatory hepatic disease [Yu and Chen, 1994], and liver injury associated with HBV infection is mediated by viral factors in addition to the host immune response. Patients who are positive for the hepatitis B e antigen (HBeAg) commonly have increased hepatic inflammatory activity and an increased risk of developing HCC [Yang et al., 2002]. HBeAg-negative HBsAg carriers who retain high levels of HBV-DNA and show persistent necroinflammation of the liver have an increased risk of acquiring HCC [Yu et al., 2005; Chen et al., 2006].

The authors report no conflicts of interest.

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Alanine aminotransferase (ALT) activity is the most widely used laboratory test for the evaluation of necroinflammatory activity in liver disease [Prati et al., 2002]; however, it is well known that HCC occurs in some HBsAg carriers with normal ALT values. Recently, Chen et al. [2006] conducted a large cohort study in Taiwan and found that elevated serum HBV-DNA levels are strong predictive factors for the development of HCC, independent of the ALT values. It is an important problem for early detection of HCC that general practitioners are sometimes unaware of those patients with normal ALT as high-risk subjects for HCC. There is little information about how many patients with normal ALT develop HCC. It is important that ALT values should be expressed with integration values to ensure a valid analysis, since ALT values fluctuate frequently [Kumada et al., 2007]. Therefore, this study sought to identify the independent risk factors, involving mainly serological markers, associated with the development of HCC in patients infected chronically with HBV with average ALT integration values ≤40 IU/L.

MATERIALS AND METHODS

Patient Selection

A total of 1,861 consecutive patients who were positive for HBsAg visited the Department of Gastroenterology at Ogaki Municipal Hospital, Japan, between September 1994 and August 2003. After assessing each patient's long-term prognosis, 381 consecutive patients were selected for further study who (1) were positive for HBsAg for at least 6 months; (2) displayed no evidence of HCV infection; (3) had no other possible causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) had a follow-up period of >3 years; (5) had no evidence of HCC for at least 3 years from the start of the follow-up period; (6) had no history of therapy involving interferons, nucleosides, or nucleotide analogues; (7) had ALT measurements taken more than twice in a year; and (8) had average ALT integration values ≤40 IU/L (Fig. 1).

Patients were evaluated at the hospital at least every 6 months. During each follow-up examination, platelets, ALT, aspartate aminotransferase (AST), gamma glutamyl transpeptidase (gamma-GTP), total bilirubin, cholinesterase, alkaline phosphatase (ALP), albumin, total cholesterol, HBeAg, anti-HBe, HBV-DNA, and alpha-fetoprotein (AFP) were measured at least every 6 months. Commercial radioimmunoassay kits were used to test blood samples for HBsAg, HBeAg, and anti-HBe (Abbott Japan Co., Ltd, Tokyo, Japan). Before July 2001, serum HBV-DNA concentrations were monitored using the amplification-hybridization protection assay (DNA probe, Chugai-HBV; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) with a lower detection limit of ~5,000 viral genome copies/ml (3.7 log copies/ml). After August 2001, serum HBV-DNA levels were monitored using the polymerase chain reaction (PCR) (COBAS Amplicor HBV monitor test, Roche Diagnostics K.K., Tokyo, Japan) with a lower detection limit of ~400 viral genome copies/ml (2.6 log copies/ml). HBV genotyping was carried out as described previously [Kato et al., 2001]. ALT, AST, gamma-GTP, ALP, and AFP were expressed as integration values [Kumada et al., 2007]. When ALT was used as an example, the integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$ (Fig. 2). The area of a trapezoid with ALT value was calculated and the measurement interval and added the values. The

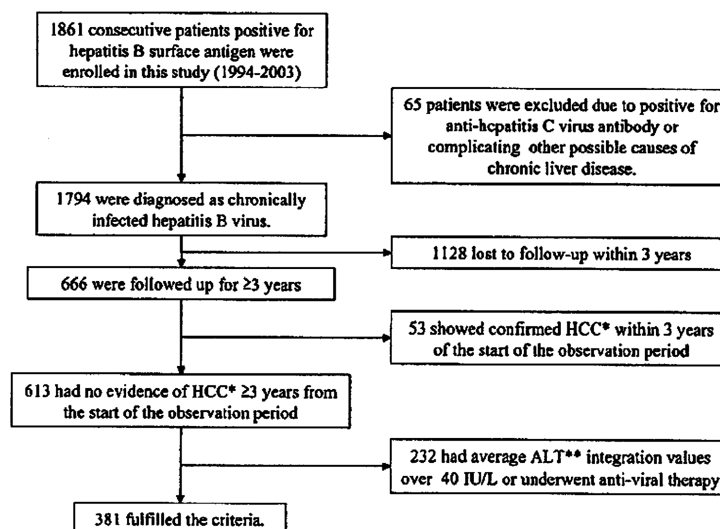

Fig. 1. Schematic flowchart of enrolled patients. *, hepatocellular carcinoma (HCC); **, alanine aminotransferase (ALT).

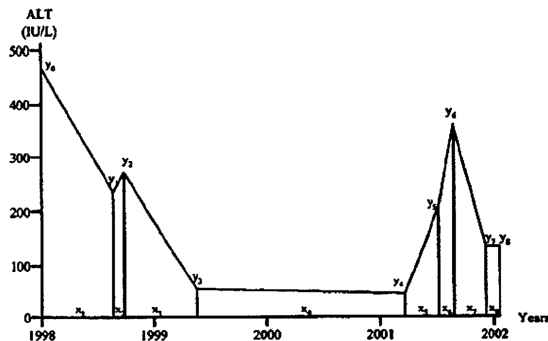

Fig. 2. Integration value of alanine aminotransferase (ALT). The integration value of ALT was calculated as follows: $(y_0+y_1)\times x_1/2+(y_1+y_2)\times x_2/2+(y_2+y_3)\times x_3/2+(y_3+y_4)\times x_4/2+(y_4+y_5)\times x_5/2+(y_5+y_6)\times x_6/2+(y_6+y_7)\times x_7/2+(y_7+y_8)\times x_8/2$. The integration value of ALT was divided by the observation period and expressed as an average integration value.

integration value of ALT was divided by the observation period to obtain the average integration value (Fig. 3). In addition, patients were classified into two groups according to the change of pattern of ALT: persistently normal ALT group and intermittently normal ALT group. The persistently normal ALT group included patients with persistently normal ALT values ≤40 IU/L during follow-up period. The intermittently normal ALT group included patients with temporary ALT fluctuations but the average integration value was ≤40 IU/L. Platelet counts, total bilirubin, cholinesterase, albumin, total cholesterol, HBeAg, anti-HBe, and HBV-DNA were analyzed at the time of entry into the study.

Ultrasonograpy was performed in all patients at the start of the follow-up period for the evaluation of liver fibrosis. The diagnosis of cirrhosis was made according to typical ultrasound findings, for example, superficial nodularity, a coarse parenchymal echo pattern, and

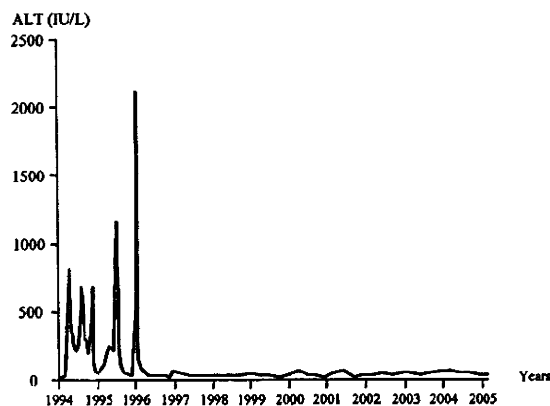

Fig. 3. Average integration value and arithmetic mean value of alanine aminotransferase (ALT) in a 26-year-old patient with hepatitis B virus (HBV). The patient was followed-up for 11.2 years. The number of ALT examinations was 96. The integration value of ALT was 955.2 IU/L × years. The average integration value was 85.3 IU/L, whereas the arithmetic mean value was 255.6 IU/L. This difference is due to the number of ALT measurements between a period of high ALT level and low ALT level.

signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [Caturelli et al., 2003; Iacobellis et al., 2005; Shen et al., 2006].

To detect early-stage HCC, ultrasonography, computed tomography, magnetic resonance imaging, and/or measurement of tumor markers (i.e., AFP, *Lens culinaris* agglutinin-reactive AFP, and des-γ-carboxyprothrombin) were performed for all patients, at least every 6 months. Blood biochemistry data used in this study were obtained over 1 year prior to HCC development. The study ended in December 31, 2007 or on the date of HCC identification, whichever was earlier. The diagnosis of HCC was based on histological examination (n = 9). In the remaining eight patients, the diagnosis was based on clinical criteria [Kudo, 1999; Torzilli et al., 1999].

Statistical Analysis

Statistical analyses were performed using the Statistical Program for Social Science (SPSS version 17.0 for Windows; SPSS Japan, Inc., Tokyo, Japan). Continuous variables are expressed as median (range). The Kruskal–Wallis test was used to assess continuous variables with a skewed distribution, and the chi-square test was used to assess categorical variables. An actuarial analysis of the cumulative incidence of HCC was performed using the Kaplan–Meier method, and differences were tested by a log-rank test. The Cox proportional hazard model and forward selection method were used to estimate the relative risk of HCC development associated with age (i.e., ≤40 years or >40 years), sex (i.e., male or female), HBeAg (i.e., positive or negative), HBV-DNA level (i.e., <5.0 or ≥5.0 log copies/ml), average ALT integration value (i.e., ≤20 or >20 IU/L), the change pattern of ALT (persistently normal ALT group or intermittently normal ALT group), average AST integration value (i.e., ≤40 or >40 IU/L), platelet count (i.e., <15.0 or ≥15.0 × 10^4/mm^3), average gamma-GTP integration value (i.e., ≤56 or >56 IU/L), total bilirubin (i.e., ≤1.2 or >1.2 mg/dl), average ALP integration value (i.e., ≤338 or >338 IU/L), cholinesterase (i.e., <431 or ≥431 IU/L), albumin (i.e., <3.5 or ≥3.5 g/dl), total cholesterol (i.e., <130 or ≥130 mg/dl), and average AFP integration value (i.e., ≤10 or >10 ng/ml). The lower and upper limits of the reference values at our institution were used as cut-off values for AST, platelet count, gamma-GTP, total bilirubin, ALP, cholinesterase, albumin, and total cholesterol. Statistical significance was defined as $P < 0.05$.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital and performed in compliance with the Helsinki Declaration.

RESULTS

Patient Characteristics

The median follow-up period was 8.6 years (range, 3.0–14.0 years). HCC developed in 17 of 381 patients

(4.5%) during the follow-up period. The 5- and 10-year cumulative incidence of HCC was 0.8% and 6.5%, respectively. Profiles and data from the 381 patients with normal ALT values are summarized in Table I.

Factors Associated With the Incidence of HCC

Factors associated with the incidence of HCC, as determined by univariate analysis, are listed in Table II. Male sex, high HBV-DNA levels, intermittently normal ALT, high AST levels, low platelet counts, low cholinesterase levels, low albumin levels, low total cholesterol levels, high AFP levels, and presence of cirrhosis were significantly associated with HCC development. The cumulative incidence of HCC was significantly higher in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70) than in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311, $P < 0.001$, Fig. 4). The cumulative incidence of HCC was significantly higher in patients with HBV-DNA levels ≥ 5.0 log copies/ml (n = 90) than in patients with HBV-DNA levels <5.0 log copies/ml (n = 291, $P < 0.001$, Fig. 5).

Factors associated with incidence of HCC, as determined by the Cox proportional hazard model and the forward selection method, are listed in Table III. Male sex, high HBV-DNA levels, low platelet counts, and low total cholesterol levels were significantly associated with the development of HCC.

Baseline of patients with normal ALT according to HBV-DNA level and platelet counts.

HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts $<15.0 \times 10^4/mm^3$ [n = 45]; C: HBV-DNA levels ≥ 5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/mm^3$ [n = 54]; and D: HBV-DNA levels ≥ 5.0 log copies/ml and platelet counts $<15.0 \times 10^4/mm^3$ [n = 25]). Positive rates of HBeAg were highest in Group C, total cholesterol levels were lowest in Group D, and ALT level, frequency of intermittently normal ALT, AFP levels, and presence

TABLE I. Patient Characteristics

Age (years)	49 (12–84)
Sex (F/M)	201/180
BMI (kg/m^2)	22.4 (17–36)
HBV genotype (A/B/C/D)	8/24/149/2
HBeAg (positive/negative)	59/322
HBV-DNA (log copies/ml)	3.7 (2.6–9.6)
ALT (IU/L)	22.6 (8.7–39.9)
Persistently normal ALT (+/−)[a]	182/199
AST (IU/L)	23.4 (13.3–74.3)
Platelet ($\times 10^4/mm^3$)	19.3 (3.3–39.5)
Gamma-GTP (IU/L)	19.5 (7.4–441.0)
Total bilirubin (mg/dl)	0.6 (0.3–4.7)
ALP (IU/L)	214.8 (82.4–621.3)
Cholinesterase (IU/L)	314.0 (99.6–483.9)
Albumin (g/dl)	4.2 (2.4–4.9)
Total cholesterol (mg/dl)	186.5 (102.0–332.1)
AFP (ng/ml)	2.4 (0.8–303.6)
Cirrhosis (−/+)[b]	341/40
Hepatocarcinogenesis (+/−)	17/364

F, female; M, male; BMI, body mass index; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase: AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; ALP, alkaline phosphatase; AFP, alpha-fetoprotein.
Values are expressed as median (range).
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound findings.

TABLE II. Factors Associated With Hepatocarcinogenesis (Univariate Analysis)

	Hazard ratio (95% CI)	*P*-value
Sex		
F	1	
M	8.282 (1.892–36.259)	0.005
HBV-DNA (log copies/ml)		
≤5.0	1	
>5.0	7.133 (2.699–18.852)	<0.001
Persistently normal ALT[a]		
Presence	1	
Absence	3.939 (1.126–13.776)	0.032
AST (IU/L)		
≤40	1	
>40	4.046 (1.157–14.140)	0.029
Platelets ($\times 10^4/m^3$)		
≥15	1	
<15	7.961 (2.922–21.690)	<0.001
Cholinesterase (IU/L)		
≥431	1	
<431	4.865 (1.368–17.298)	0.015
Albumin (g/dl)		
≥3.5	1	
<3.5	8.086 (2.567–25.474)	<0.001
Total cholesterol (mg/dl)		
≥130	1	
<130	9.704 (2.740–34.367)	<0.001
AFP (ng/ml)		
≤10	1	
>10	6.779 (1.445–31.809)	0.015
Cirrhosis[b]		
Absence	1	
Presence	18.033 (6.6055–19.233)	<0.001

W, female; M, male; HBV, hepatitis B virus; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase; AFP, alpha-fetoprotein.
P-values and hazard ratio were calculated by Cox proportional hazard model.
[a]Persistently normal ALT values includes patients with ≤40 IU/L.
[b]Cirrhosis diagnosed by ultrasound.

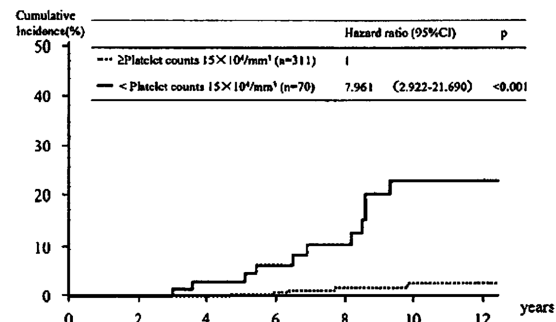

Fig. 4. Incidence of HCC according to platelet counts. The 5- and 10-year cumulative incidences of HCC was 0.4% and 2.6%, respectively, in patients with platelet counts $\geq 15.0 \times 10^4/mm^3$ (n = 311), and 2.9% and 22.9% in patients with platelet counts $<15.0 \times 10^4/mm^3$ (n = 70). The cumulative incidence of HCC was significantly higher in the latter group than in the former.

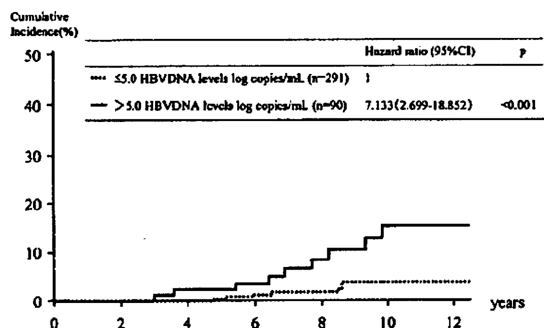

Fig. 5. Incidence of HCC according to serum HBV-DNA levels. The 5- and 10-year cumulative incidences of HCC was 0.4% and 3.7%, respectively, in patients with HBV-DNA levels <5.0 log copies/ml (n = 291) and 2.3% and 15.5%, respectively, in patients with HBV-DNA levels ≥5.0 log copies/ml (n = 90). The cumulative incidence of HCC was significantly higher in the latter group than in the former.

of cirrhosis were highest in Group D (Table IV). Group D showed the highest rate of incidence of HCC, followed by Groups B and C, as compared with Group A (Fig. 6).

DISCUSSION

The current studies revealed that the risk of developing HCC increases with decreasing platelet counts, decreasing total cholesterol levels, and increasing HBV-DNA levels in patients with average ALT integration values ≤40 IU/L.

ALT, AST, gamma-GTP, ALP, and AFP levels fluctuated within individual patients. Therefore, repeated measurements of these tests are important for accurate interpretation of the data. The arithmetic mean value is often used in the measurement of these tests; however, this value can be greatly affected by the period of time between measurements. Therefore, integral calculus was used to determine the value of these markers. Because this determination is strongly affected by the follow-up period, the average integration value was divided by the time of follow-up. The average integration

TABLE III. Multivariate Analysis of Factors Associated With Development of Hepatocellular Carcinoma

Factor	Hazard ratio (95% CI)	P-value
Sex		
F	1	
M	6.011 (1.353–26.710)	0.018
HBV-DNA (log copies/ml)		
≤5.0	1	
>5.0	5.125 (1.880–13.973)	0.001
Platelets ($\times 10^4$/mm^3)		
≥15	1	
<15	4.803 (1.690–13.647)	0.003
Total cholesterol (mg/dl)		
≥130	1	
<130	5.983 (1.558–22.979)	0.009

F, female; M, male; HBV, hepatitis B virus.
P-values and hazard ratios were calculated using the Cox proportional hazard model.

value is more meaningful than the arithmetic mean value [Kumada et al., 2007].

In the present study, there was no difference between patients with average ALT integration values of 0–20 IU/L versus those with 21–40 IU/L. Thus, ALT levels are not good predictors of HCC development in patients with hepatitis B, as opposed to hepatitis C [Yuen et al., 2005; Sherman, 2005]. Furthermore, the change pattern of ALT was evaluated in the persistently normal ALT group and the intermittently normal ALT group. The results of the univariate analysis suggest that intermittently normal ALT levels, high AST levels, low cholinesterase levels, low albumin levels, and high AFP levels are associated significantly with HCC development; however, not all of these factors were significant in the multivariate analysis.

HBV-DNA levels at the start of the follow-up period correlated with the cumulative incidence of HCC. Chen et al. [2006] reported the adjusted hazard ratios for HCC development in HBeAg-seronegative subjects with normal ALT levels. Compared with participants in whom serum HBV-DNA levels were <300 copies/ml, the adjusted hazard ratio for developing HCC was 1.3 (95% confidence interval, 0.5–3.2; $P = 0.05$) for participants with serum HBV-DNA levels of 300–9,999 copies/ml; 2.7 (1.2–6.3; $P = 0.02$) for levels of 10,000–99,999 copies/ml; 7.2 (3.2–16.6; $P < 0.001$) for levels of 100,000–999,999 copies/ml; and 14.3 (6.2–32.8; $P < 0.001$) for levels of 1 million copies/ml and greater. It is emphasized that the cumulative incidence of HCC increases in patients with increased HBV-DNA levels, even if patients have normal ALT levels.

Lok and McMahon [2004] reported that HBV-DNA levels $>10^5$ copies/ml should be considered clinically significant. Their recommendation is supported by a meta-analysis of 26 trials of anti-HBV therapy which evaluated the association between viral load and hepatic inflammatory activity, as determined by hepatic histology and aminotransferase activity [Mommeja-Marin et al., 2003]. Thus, it is important for patients to maintain low HBV-DNA levels (i.e., $\leq 10^5$ copies/ml). These findings suggest that effective control of HBV replication, indicated by a decrease in serum HBV-DNA levels following antiviral therapy, may reduce the ultimate risk of developing HCC. Furthermore, it is believed that treatment with nucleosides or nucleotide analogues will decrease the cumulative incidence of HCC [Liaw et al., 2004; Piao et al., 2005].

The present study reveals that a low platelet count is a predictive factor for the development of HCC. Cirrhosis is an established risk factor for HCC in patients with HBV [Liaw et al., 1989; McMahon et al., 2001; Yu et al., 2002; Murata et al., 2005]. Ultrasonography produces detailed cross-sectional images of the liver and its surrounding structures. To distinguish cirrhosis patients from non-cirrhosis patients was attempted according to typical ultrasound findings [Caturelli et al., 2003; Iacobellis et al., 2005; Shen et al., 2006]. The presence of cirrhosis diagnosed by ultrasonography

TABLE IV. Patients Characteristics, According to HBVDNA Levels and Platelet Counts

HBV-DNA (log copies/ml) Platelets ($\times 10^4$/m^3)	Group A $\leq$5.0 $\geq$15 × 10^4 (n = 257)	Group B $\leq$5.0 <15 × 10^4 (n = 45)	Group C >5.0 $\geq$15 × 10^4 (n = 54)	Group D >5.0 <15 × 10^4 (n = 25)
Age (years)	49 (12–84)	51 (24–75)	47 (15–73)	52 (33–82)
Sex (F/M)	136/121	25/20	29/25	11/14
BMI (kg/m^2)	22.6 (14–36.3)	22.5 (16–28.2)	22.2 (16.7–32.4)	20.9 (16.9–36.4)
HBV genotype (A/B/C/D)	7/20/88/2	0/1/20/0	1/3/26/0	0/0/15/0
HBeAg (positive/negative)***	5/252	3/42	36/18	15/10
ALT (IU/L)***	19.7 (8.7–39.1)	25.3 (11.2–38.2)	29.8 (12.2–39.9)	32.1 (18.3–38.4)
Persistently normal ALT (+/−)[a],***	153/104	14/31	14/40	1/24
Total cholesterol (mg/dl)***	191.5 (114–332.1)	169.1 (102–259.2)	190.1 (147.1–254.4)	165.5 (112–234)
AFP (ng/ml)****	2.2 (0.8–119.8)	2.6 (0.8–20.8)	2.8 (0.8–45.5)	4.7 (1.1–303.6)
Cirrhosis (−/+)[b],***	253/4	27/18	50/4	11/14
Hepatucelluar carcinoma (+/−)***	2/255	5/40	4/50	6/19

F, female; M, male; BMI, body mass index; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; AFP, alpha-fetoprotein.
P-values were calculated using the Kruskal–Wallis test or the chi-square test. Values are expressed as median (range).
[a]Persistently normal ALT values includes patients with $\leq$40 IU/L.
[b]Cirrhosis diagnosed by ultrasound findings.
***$P < 0.0001$.
****$P < 0.0005$.

was strongly associated with the increased incidence of HCC by univariate analysis. Anatomical constraints and interobserver variability, however, remain limiting factors. In this study, histological confirmation was obtained in only 20 patients (6.3%). It is thought that this study had limitations because the liver histology was not obtained in many cases. Liver biopsy is still the "gold standard" for assessing liver fibrosis; however, it is not practical to undertake biopsies on all patients because of the potential complications which might arise from this procedure. Furthermore, results often differ depending on the pathologist, and results for liver fibrosis in liver biopsy specimens do not always reflect the grade of fibrosis in the entire liver. In contrast, the platelet count is a useful surrogate marker for the

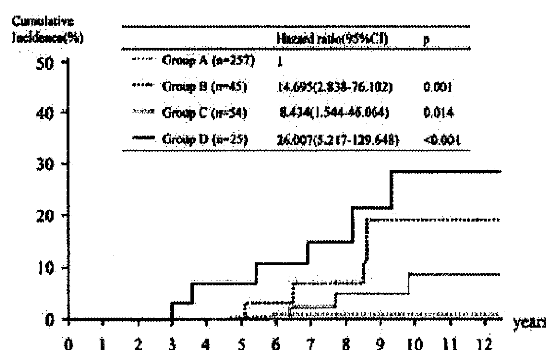

Fig. 6. The cumulative incidence of HCC according to HBV-DNA levels and platelet counts. HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels <5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4$/mm^3 [n = 257]; B: HBV-DNA levels <5.0 log copies/ml and platelet counts $<15.0 \times 10^4$/mm^3 [n = 45]; C: HBV-DNA levels $\geq$5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4$/mm^3 [n = 54]; and D: HBV-DNA levels $\geq$5.0 log copies/ml and platelet counts $<15.0 \times 10^4$/mm^3 [n = 25]). Group D had the highest incidence rate of HCC (26.007 [5.217–129.648], $P < 0.001$), followed by Group B (14.695 [2.838–76.102], $P = 0.001$) and Group C (8.434 [1.544–46.064], $P = 0.014$), as compared with Group A.

diagnosis of cirrhosis. Lu et al. [2006] reported that the best cutoff platelet count for a diagnosis of cirrhosis is 15.0×10^4/mm^3. The primary aim of this study was to identify serological markers associated with the development of HCC. Because of this, cirrhosis diagnosed by ultrasonography was excluded from the multivariate analysis. On the other hand, a low cholesterol level is associated with hepatocarcinogenesis, too. Hypocholesterolemia is found frequently in advanced liver disease because the liver is the most active site of cholesterol metabolism [D'Arienzo et al., 1998]. Four of 12 patients (33.3%) with <130 mg/dl serum total cholesterol developed HCC during follow-up period. It seemed that low platelet counts and hypocholesterolemia were confounding factors for identifying cirrhosis. Platelet counts were used as a parameter for cirrhosis in this study.

The HBV genotype is also predictive of the development of HCC [Chan et al., 2004; Yu et al., 2005]. In Japan, HBV genotype C is the predominant genotype [Orito et al., 2001]. Genotype C is associated with higher HBV-DNA levels and a greater risk of HCC than genotype B [Chan et al., 2004]. In the present study, 149 of 183 patients (81.4%) were infected with HBV genotype C. All eight patients with HCC in whom HBV genotype was determined were infected with genotype C. It was difficult to evaluate the relationship between HBV genotype and incidence of HCC in this study.

This study has some limitations such as the potential for selection bias due to a retrospective analysis of a cohort of patients. Therefore, an effort was made to minimize the influence of bias by using average integration values of various biochemical markers and a multivariate analysis.

In conclusion, high HBV-DNA levels and low platelet counts are associated with an increased incidence of HCC in patients infected with hepatitis B who have normal ALT values. Therefore, maintenance of low HBV-DNA levels is important for the prevention for

HCC in patients with low platelet counts, even when the ALT values fall within the current normal range.

REFERENCES

Beasley RP. 1988. Hepatitis B virus. The major etiology of hepatocellular carcinoma. Cancer 61:1942–1956.

Beasley RP, Hwang LY, Lin CC, Chien CS. 1981. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22707 men in Taiwan. Lancet 2:1129–1133.

Caturelli E, Castellano L, Fusilli S, Palmentieri B, Niro GA, del Vecchio-Blanco C, Andriulli A, de Sio I. 2003. Coarse nodular US pattern in hepatic cirrhosis: Risk for hepatocellular carcinoma. Radiology 226:691–697.

Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, Sung JJ. 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 53:1494–1498.

Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. 2006. REVEAL-HBV Study Group: Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 295:65–73.

D'Arienzo A, Manguso F, Scaglione G, Vicinanza G, Bennato R, Mazzacca G. 1998. Prognostic value of progressive decrease in serum cholesterol in predicting survival in Child-Pugh C viral cirrhosis. Scand J Gastroenterol 33:1213–1218.

EASL Jury. 2003. EASL International Consensus Conference on Hepatitis B. 13–14 September, 2002: Geneva, Switzerland. Consensus statement (short version). J Hepatol 38:533–540.

Iacobellis A, Fusilli S, Mangia A, Clemente R, Festa V, Giacobbe A, Facciorusso D, Niro G, Conoscitore P, Andriulli A. 2005. Ultrasonographic and biochemical parameters in the non-invasive evaluation of liver fibrosis in hepatitis C virus chronic hepatitis. Aliment Pharmacol Ther 22:769–774.

Ikeda K, Arase Y, Kobayashi M, Someya T, Hosaka T, Saitoh S, Sezaki H, Akuta N, Suzuki F, Suzuki Y, Kumada H. 2005. Hepatitis B virus-related hepatocellular carcinogenesis and its prevention. Intervirology 48:29–38.

Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Methods 98:153–159.

Kiyosawa K, Umemura T, Ichijo T, Matsumoto A, Yoshizawa K, Gad A, Tanaka E. 2004. Hepatocellular carcinoma: Recent trends in Japan. Gastroenterology 127:S17–S26.

Kudo M. 1999. Imaging diagnosis of hepatocellular carcinoma and premalignant/borderline lesions. Semin Liver Dis 19:297–309.

Kumada T, Toyoda H, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Kanamori A, Kondo J, Yamauchi T, Nakano S. 2007. Relation between incidence of hepatic carcinogenesis and integration value of alanine aminotransferase in patients with hepatitis C virus infection. Gut 56:738–739.

Liaw YF, Lin DY, Chen TJ, Chu CM. 1989. Natural course after the development of cirrhosis in patients with chronic type B hepatitis: A prospective study. Liver 9:235–241.

Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J. 2004. Cirrhosis Asian Lamivudine Multicentre Study Group. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 351:1521–1531.

Lok AS, McMahon BJ. 2004. Practice Guidelines Committee, American Association for the Study of Liver Diseases (AASLD): Chronic hepatitis B: Update of recommendations. Hepatology 39:857–861.

Lu SN, Wang JH, Liu SL, Hung CH, Chen CH, Tung HD, Chen TM, Huang WS, Lee CM, Chen CC, Changchien CS. 2006. Thrombocytopenia as a surrogate for cirrhosis and a marker for the identification of patients at high-risk for hepatocellular carcinoma. Cancer 107:2212–2222.

McMahon BJ, Holck P, Bulkow L, Snowball M. 2001. Serologic and clinical outcomes of 1536 Alaska Natives chronically infected with hepatitis B virus. Ann Intern Med 135:759–768.

Mommeja-Marin H, Mondou E, Blum MR, Rousseau F. 2003. Serum HBV DNA as a marker of efficacy during therapy for chronic HBV infection: Analysis and review of the literature. Hepatology 37:1309–1319.

Murata K, Sugimoto K, Shiraki K, Nakano T. 2005. Relative predictive factors for hepatocellular carcinoma after HBeAg seroconversion in HBV infection. World J Gastroenterol 11:6848–6852.

Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 34:590–594.

Piao CY, Fujioka S, Iwasaki Y, Fujio K, Kaneyoshi T, Araki Y, Hashimoto K, Senoh T, Terada R, Nishida T, Kobashi H, Sakaguchi K, Shiratori Y. 2005. Lamivudine treatment in patients with HBV-related hepatocellular carcinoma–using an untreated, matched control cohort. Acta Med Okayama 59:217–224.

Prati D, Taioli E, Zanella A, Della Torre E, Butelli S, Del Vecchio E, Vianello L, Zanuso F, Mozzi F, Milani S, Conte D, Colombo M, Sirchia G. 2002. Updated definitions of healthy ranges for serum alanine aminotransferase levels. Ann Intern Med 137:1–10.

Rustgi VK. 1987. Epidemiology of hepatocellular carcinoma. Gastroenterol Clin North Am 16:545–551.

Shen L, Li JQ, Zeng MD, Lu LG, Fan ST, Bao H. 2006. Correlation between ultrasonographic and pathologic diagnosis of liver fibrosis due to chronic virus hepatitis. World J Gastroenterol 28:1292–1295.

Sherman M. 2005. Predicting survival in hepatitis B. Gut 54:1521–1523.

Szmuness W. 1978. Hepatocellular carcinoma and the hepatitis B virus: Evidence for a causal association. Prog Med Virol 24:40–69.

Torzilli G, Minagawa M, Takayama T, Inoue K, Hui AM, Kubota K, Ohtomo K, Makuuchi M. 1999. Accurate preoperative evaluation of liver mass lesions without fine-needle biopsy. Hepatology 30:889–893.

Wong CH, Chan SK, Chan HL, Tsui SK, Feitelson M. 2006. The molecular diagnosis of hepatitis B virus-associated hepatocellular carcinoma. Crit Rev Clin Lab Sci 43:69–101.

Yang HI, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ. 2002. Taiwan Community-Based Cancer Screening Project Group. Hepatitis B e antigen and the risk of hepatocellular carcinoma. N Engl J Med 347:168–174.

Yu MW, Chen CJ. 1994. Hepatitis B and C viruses in the development of hepatocellular carcinoma. Crit Rev Oncol Hematol 17:71–91.

Yu MW, Chang HC, Chen PJ, Liu CJ, Liaw YF, Lin SM, Lee SD, Lin SC, Lin CL, Chen CJ. 2002. Increased risk for hepatitis B-related liver cirrhosis in relatives of patients with hepatocellular carcinoma in northern Taiwan. Int J Epidemiol 31:1008–1015.

Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, Shih WL, Kao JH, Chen DS, Chen CJ. 2005. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: A prospective study in men. J Natl Cancer Inst 97:265–272.

Yuen MF, Yuan HJ, Wong DK, Yuen JC, Wong WM, Chan AO, Wong BC, Lai KC, Lai CL. 2005. Prognostic determinants for chronic hepatitis B in Asians: Therapeutic implications. Gut 54:1610–1614.