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Introduction

Primary squamous cell carcinoma (SCC) of the liver is a rare tumor which has been sporadically reported. To our knowledge, only 24 cases have been reported in the English language literature. Of those cases, many developed in livers accompanied by nonparasitic hepatic cysts [1–5]. Solitary and multiple nonparasitic cysts originate from von Meyenburg's complexes and are lined with simple cuboidal or columnar epithelium. Primary SCC of the liver is generally considered to result from secondary squamous metaplasia of such biliary epithelium due to chronic inflammation and subsequent neoplastic transformation [3]. The prognosis of this tumor is very poor, and only a few cases survived more than 12 months even after treatment [6, 7]. In addition to surgical resection, radiation and chemotherapy have been tried to improve survival [8, 9]. We report here an advanced case of primary SCC of the liver who received surgical resection and subsequent chemotherapy with low-dose 5-fluorouracil (5-FU) and cisplatin (CDDP). In addition, we reviewed in the literature which kind of treatment is appropriate if hepatic tumors are diagnosed as primary SCC.

Contrast-enhanced ultrasonography (CE-US) is a useful method for diagnosis of hepatic tumors. While findings of CE-US in hepatocellular carcinoma, cholangiocellular carcinoma, or metastatic adenocarcinoma of the liver have been well documented, there is no report of CE-US findings in primary hepatic SCC so far. CE-US findings of primary SCC of the liver were also reported here for the first time.

Case Report

A 73-year-old Japanese woman with a liver tumor and right hypochondrial pain was referred to our department in September 2006 for the purpose of surgical treatment, while her chief complaint started in June 2006. She visited a former hospital, and a liver tumor was suspected in the right lobe. Routine laboratory tests were within normal range. Enhanced computed tomography (CT) demonstrated an irregular low-density area with marginal enhancement adjacent to a nonparasitic liver cyst (fig. 1), while another nonparasitic cyst was also detected in segment 6. Comparison between enhanced CT images in June 2006 (fig. 1a) and those in September 2006 (fig. 1b) revealed that invasion of this liver tumor into right diaphragm and lung tissue became obvious during this period. Magnetic resonance imaging (MRI) demonstrated an irregular liver tumor with low signal intensity in the T1-weighed images and slightly high signal intensity in the T2-weighed images (data not shown). Diffusion-weighted image showed abnormal intensity in the tumor adjacent to the nonparasitic cyst. Ultrasonography (US) revealed an irregular hyperechoic lesion in segments 7 and 8 in the liver (fig. 1c). CE-US using Levovist® (Berlex, Canada) showed blood flow into the tumor from the marginal area during the early vascular phase (fig. 1c; 23 s after injection). The increased tumor blood flow lasted during the late vascular phase except for the central area (fig. 1c; 60 s); then, delayed parenchymal phase of the US showed an irregular hypoechoic area (fig. 1c; 5 min). Meanwhile, whole body CT and gastrointestinal endoscopy demonstrated no other extrahepatic tumors. From these image analyses, intrahepatic cholangiocarcinoma or cyst adenocarcinoma in the liver was preoperatively suspected, even though the CE-US finding of the tumor in the late vascular phase was atypical. Needle biopsy was not performed to prevent iatrogenic dissemination.

In October 2006, she underwent hepatic resection of segments 7 and 8 including the nonparasitic cyst, in combination with partial resection of right diaphragm and right lung tissues. The tumor was irregular and white-colored, and its size was 10.0 × 8.0 cm (fig. 2). The tumor macroscopically seemed to form a part of the nonparasitic cyst wall, invading to the liver, diaphragm and lung tissues. Histological examination revealed that the tumor consisted of moderately or partially well differentiated squamous carcinoma cells with keratinization, but not of any adenocarcinoma component (fig. 3a–c). Invasion of the tumor cells into the lung tissue was also microscopically detected (fig. 3a, asterisk). Immunohistochemical analysis revealed that this tumor contained many capillary endothelial vessels

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which were positive for anti-CD31 (fig. 3d) or anti-CD34 (fig. 3e) antibodies, and that these vessels predominantly existed in the peripheral regions of the tumor. Meanwhile, the tumor was negative for immunostaining against thyroid transcription factor-1 (TTF-1), a pulmonary cell-selective transcription protein (data not shown).

After the operation, the patient underwent systemic chemotherapy with low-dose 5-FU (250 mg/kg body weight/day) and CDDP (5 mg/kg body weight/day). These drugs were administered for 5 consecutive days with 2-day intervals for 4 weeks. Meanwhile radiation therapy could not be performed because of postoperative bleeding peptic ulcer. Four months after the operation, enhanced CT images revealed no recurrence in the remnant liver and surrounding tissues. However, recurrence appeared 11 months later, and she died 13 months after the operation.

Discussion

Primary SCC of the liver is a very rare tumor, and many of them exist adjacent to nonparasitic liver cysts, with a few exceptions [7, 10]. While the precise mechanism why SCC develops in the liver is unclear, it is proposed that squamous epithelium lining liver cysts may undergo dysplasia, metaplasia and, ultimately, malignant transformation over the years [3]. In the present case, the SCC indeed existed adjacent to a nonparasitic cyst, forming a part of the cyst wall, suggesting that the tumor originated from the cyst wall. Strictly, it is difficult to distinguish primary SCC of the liver from hepatic invasion of pulmonary SCC in the present case, though the tumor was negative for anti-TTF-1 immunostaining. However, sequential preoperative image analysis strongly suggested that primary SCC of the liver invaded into the right diaphragm and lung tissue in this case.

In the diagnosis of primary hepatic SCC, preoperative findings of this tumor in CE-US have not been well defined in the literature. In the present case, the tumor was still hyperechoic with Levovist even in the late vascular phase. This observation was incompatible with typical CE-US findings of cholangiocarcinoma, metastatic adenocarcinoma, or hepatocellular carcinoma [11]. Meanwhile, CT and MRI images of the present tumor were almost compatible with those in previous hepatic SCC cases [7, 10, 12]. Immunohistochemical analysis (anti-CD31 or anti-CD34) revealed abundance of capillary endothelial vessels in this tumor. This observation may explain why the tumor showed hyperechoic findings even in the late vascular phase during CE-US, as reported in a case of cholangiolocellular carcinoma [13]. Collecting CE-US data concerning SCC will be required to define whether the present observation is specific to SCC or not. Preoperative needle biopsy might be recommended if a rare pattern of CE-US is preoperatively detected like in the present case, because the therapeutic strategy will be possibly changed due to the pathological diagnosis.

As for the treatment of primary SCC of the liver, surgical resection per se can lead to a good prognosis if the tumor stays within early stages, namely if the tumor invades only the subepithelial connective tissue of the cyst wall, but not the surrounding liver parenchyma [14, 15]. However, primary SCC is often diagnosed after it has become advanced. In such cases, the tumor invades beyond the cyst wall and the liver parenchyma, leading to very poor prognosis. Before 1994, no case with survival >6 months was reported in such advanced patients [15]. Thereafter, radiation therapy or chemotherapy was tried to improve the prognosis, and relatively good survival was reported in a few cases [6–9]. We summarized which kind of therapy was applied to the patients with primary SCC of the liver when relatively good survival (>12 months) was reported in the English language literature (table 1). From the summary, it is

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suggested that surgical resection alone cannot promise good prognosis once the tumor has become advanced, and that radiation therapy or chemotherapy with 5-FU and/or CDDP should be recommended in combination with surgical resection. In the present case, the diagnosis of hepatic SCC was confirmed only after liver resection, and postoperative systemic chemotherapy with 5-FU (250 mg/kg body weight/day) and CDDP (5 mg/kg body weight/day) was performed for 4 weeks (administration for 5 consecutive days with 2-day intervals). However, she did not undergo radiation therapy because of postoperative bleeding peptic ulcer. She died 13 months after the operation because of recurrence. These observations strongly suggest that radiation therapy in combination with chemotherapy and surgery should be applied if the hepatic tumor appears to be advanced primary SCC.

In summary, although primary SCC of the liver is very rare, if atypical hepatic tumor exists adjacent to a nonparasitic liver cyst, SCC also should be taken into the differential diagnosis. At present, there are no guidelines for adjuvant and palliative chemotherapy for primary SCC of the liver. However, it can be proposed that combination of radiation therapy, systemic chemotherapy, and surgical resection, if possible, should be applied if hepatic tumors are preoperatively confirmed as primary SCC.

Disclosure Statement

Yuji Iimuro and the co-authors have no conflict of interest.

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Table 1. Primary SCC of the liver: literature review of treatment in patients with relatively good survival (>12 months)

Reference	Age, sex	Clinical stage	Therapy	Survival
Banbury et al., 1994 [14]	59, female	not advanced	surgery only	>16 months
Weimann et al., 1996 [15]	74, female	not advanced	surgery only	>4 years
Kaji et al., 2003 [12]	67, female	advanced	hepatic arterial infusion of CDDP and 5-FU	23 months
Hsieh et al., 2005 [8]	65, male	advanced	surgery + systemic 5-FU + radiation	18 months
Boscolo et al., 2005 [9]	64, male	advanced	systemic CDDP and 5-FU + surgery	complete remission
Abbas et al., 2008 [6]	28, female	advanced	surgery + radiation	>18 months
Naik et al., 2009 [7]	56, male	advanced	radiation + surgery	>6 years

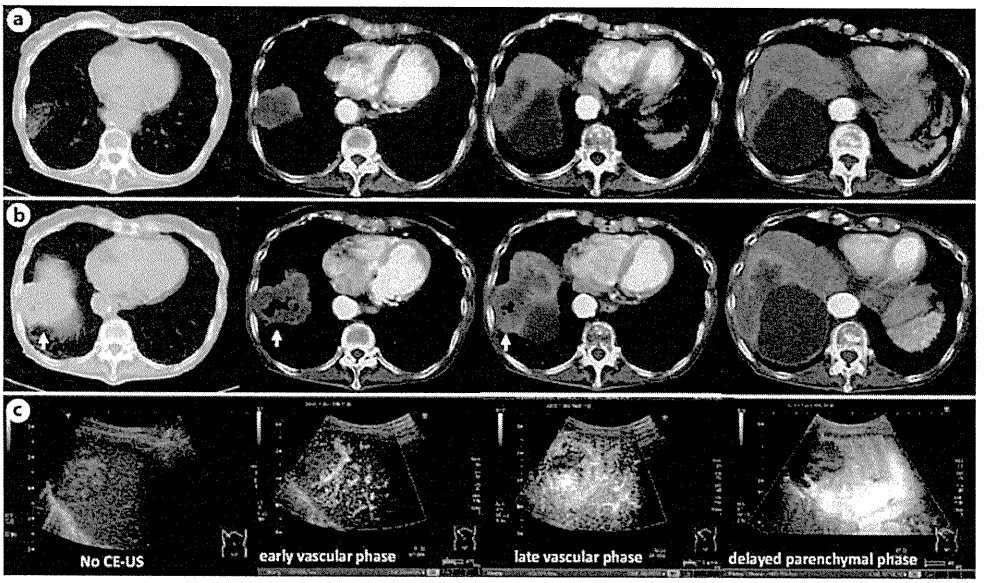

Fig. 1. Plain chest or enhanced abdominal CT on June 2006 (**a**) and September 2006 (**b**) of the hepatic tumor are shown. Comparison between these two series of CT images revealed that an irregular low-density hepatic tumor with marginal enhancement located adjacent to a nonparasitic liver cyst gradually grew and invaded into the diaphragm and the lung tissue. Arrows: lung tissues involved in the tumor. Abdominal US revealed an irregular hyperechoic lesion in segments 7 and 8 of the liver (**c**). CE-US demonstrated blood flow into the tumor from the periphery in the early vascular phase (23 s after the injection of Levovist). The contrast enhancement lasted until the late vascular phase (60 s) except for the central area (necrotic lesion). The delayed parenchymal phase of the US (5 min) showed an irregular hypoechoic area.

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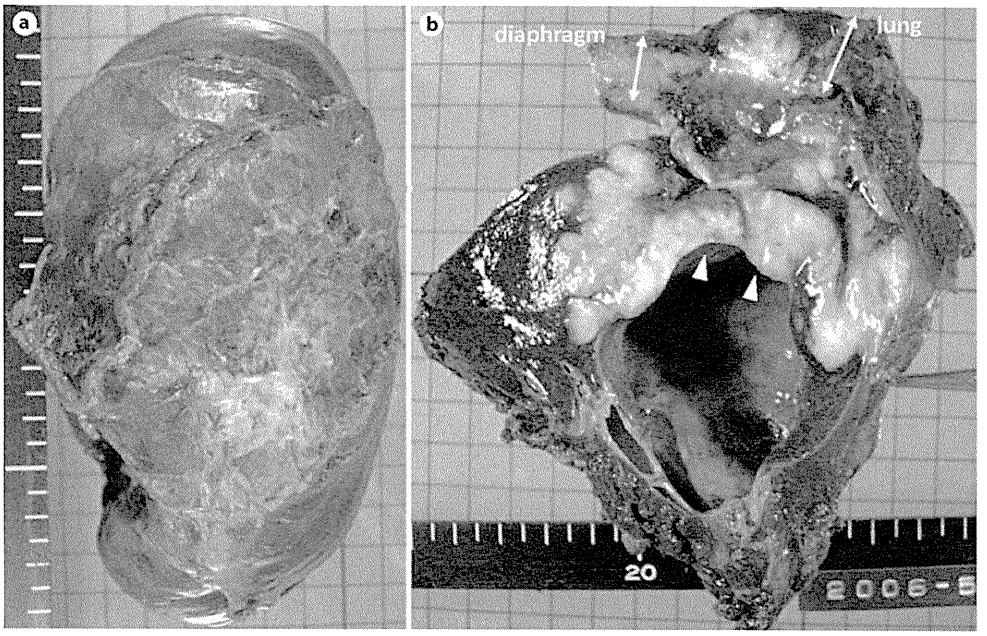

Fig. 2. Macroscopic findings of the resected tumor including diaphragm and lung tissue are shown (**a**). Coronal section of the specimen revealed that the tumor was irregular and white-colored, forming a part of the nonparasitic cyst wall, invading to the liver, diaphragm and lung tissue (**b**).

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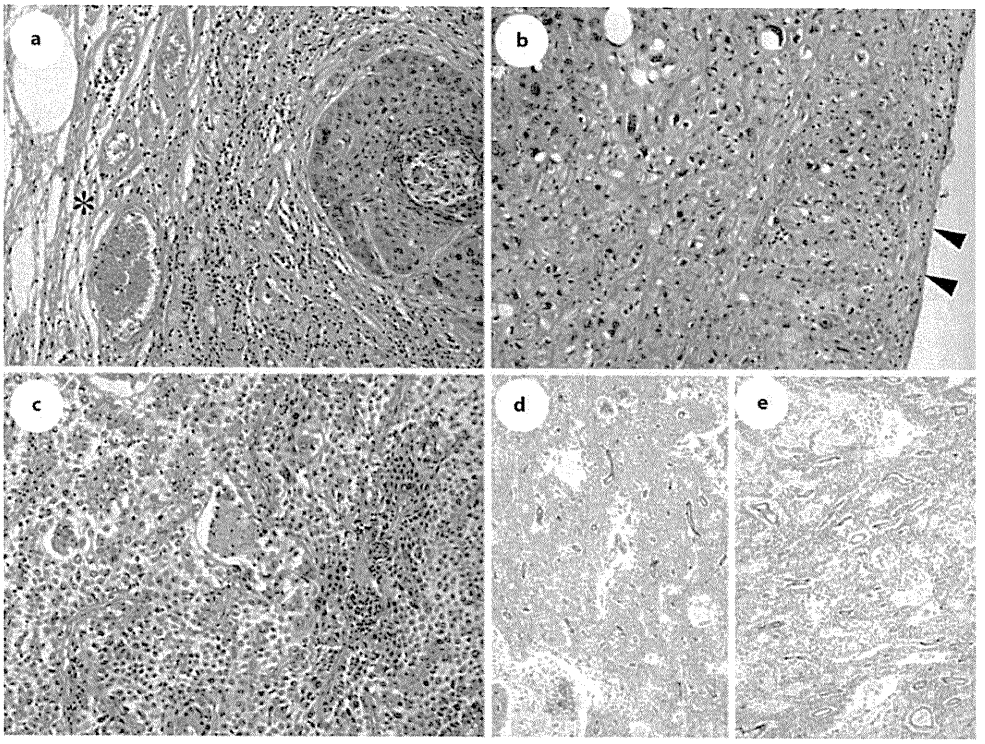

Fig. 3. Histological examination revealed that the tumor consisted of moderately (**b**, **c**) or partially well (**a**) differentiated squamous carcinoma cells with keratinization. Invasion of the tumor cells into the lung tissue was microscopically detected (**a**). Meanwhile, no adenocarcinoma component was detected in the specimen. Immunohistochemical analysis revealed that this tumor contained many capillary endothelial vessels which were positive for anti-CD31 (**d**) or anti-CD34 (**e**) antibodies. Asterisk: compressed lung tissue; arrowheads: wall of the nonparasitic cyst. H&E staining: original magnification ×200; immunostainings: original magnification ×100.

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Original Article

Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B

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Aim: The factors associated with hepatitis recurrence after discontinuation of nucleos(t)ide analogs (NAs) in patients with chronic hepatitis B were analyzed to predict the risk of relapse more accurately.

Methods: A total of 126 patients who discontinued NA therapy were recruited retrospectively. The clinical conditions of a successful discontinuation were set as alanine aminotransferase (ALT) below 30 IU/L and serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL.

Results: Relapse of hepatitis B were judged to occur when maximal serum ALT became higher than 79 IU/L or when maximal serum HBV DNA surpassed 5.7 log copies/mL following NA discontinuation since these values corresponded with mean values of ALT (30 IU/L) and HBV DNA (4.0 log copies/mL), respectively. At least 90% of patients with either detectable hepatitis B e antigen or serum HBV DNA higher than 3.0 log copies/mL at the time of NA discontinuation relapsed within one year. In the remaining patients, higher levels of both hepatitis B surface and core-related antigens at the time of discontinuation, as well as a shorter course of NA treatment, were significantly associated with relapse by multivariate analysis.

Conclusions: It appears that negative results for hepatitis B e antigen and serum HBV DNA lower than 3.0 log copies/mL are essential for successful NA discontinuation, which may be attained by a longer treatment period. Levels of hepatitis B surface and core-related antigens are also significant factors independently associated with relapse of hepatitis.

Key words: discontinuation, hepatitis B core-related antigen, hepatitis B surface antigen, nucleos(t)ide analogs, relapse of hepatitis

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INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, and may eventually develop into liver cirrhosis and hepatocellular carcinoma.[1–3] Over the last decade, major advances in the treatment of chronic hepatitis B have been made with nucleos(t)ide

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analogs (NAs) such as lamivudine (LVD), adefovir dipivoxil (ADV), and entecavir (ETV).[4] NAs are orally administered and are associated with low rates of adverse effects. Treatment with NAs shows strong suppression of HBV replication and consequently rapid improvement of elevated ALT levels. Furthermore, these drugs have been reported to lower the risk of complicating cirrhosis and hepatocellular carcinoma,[5–7] and so NAs are becoming widely used to treat patients with chronic hepatitis B. On the other hand, NAs carry the risk of developing drug-resistance;[8] drug-resistant viruses emerging during treatment may be associated with hepatitis flare-ups. Hepatitis B patients are also required to undergo prolonged treatment with NAs because early discontinuance often leads to relapse of hepatitis and ensuing hepatic failure following rises in alanine aminotransferase (ALT) level.[9,10]

Serum HBV DNA is normally used to monitor the antiviral effect of NAs. HBV DNA decreases rapidly and becomes undetectable in the majority of patients who are treated with NAs,[11–13] but relapse after discontinuation is not rare.[14–17] Since it is also true that favorable virological and biochemical responses to NAs may continue indefinitely in some patients,[9,15] reliable markers that can predict relapse of hepatitis after NA discontinuation are needed. Such markers would benefit not only patients who are considering discontinuation of NA treatment, but also clinicians, hospitals, and the medical economy.

In the present study, we assessed several factors associated with relapse of hepatitis after discontinuation of NAs in patients with chronic hepatitis B, including hepatitis B viral antigens, which have been reported as new and promising markers for monitoring the effect of antiviral agents, such as interferon and NAs.

METHODS

Patients

A TOTAL OF 126 patients with chronic hepatitis B who underwent and completed NA treatment between 2000 and 2010 were enrolled in this study. Patients were recruited retrospectively from 11 hospitals across Japan (Toranomon Hospital, Hokkaido University Hospital, Nagoya City University Hospital, Shinshu University Hospital, Hiroshima University Hospital, National Hospital Organization Nagasaki Medical Center, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Japanese Red Cross Nagoya Daini Hospital, and Tokyo Women's Medical University Hospital, Sapporo Kosei General Hospital) and met the following conditions: (i) serum ALT higher than 30 IU/L and serum HBV DNA higher than 4.0 log copies/mL were observed at least twice within the 6 months prior to administration of NAs; (ii) stored serum samples at initiation and discontinuation of NAs were available for measurements of viral markers; (iii) clinical outcomes were followed for at least 6 months after the discontinuation of NAs; and (iv) tests for hepatitis C and human immunodeficiency virus antibodies were negative. Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions at least 6 months apart in all patients before treatment. Patients complicated with hepatocellular carcinoma or signs of hepatic failure at treatment discontinuation were excluded from the study. Our cohort consisted of 83 men and 43 women with a median age of 46 (range, 19 to 79) years when NA administration was discontinued. Hepatitis B e antigen (HBeAg) was positive in 64 patients (51%) at the initiation of treatment and in 24 patients (19%) at its discontinuation. HBV genotype was A in two (2%) patients, B in five (4%), C in 102 (81%), and undetermined in 17 (13%). Thirty-five of the 126 patients in this study were younger than 35 years old. Although not recommended as the first line treatment for this group by Japanese guidelines,[18] NA treatment was commenced since chronic active hepatitis had been persisting in all cases irrespective of their HBeAg status (26 positive and nine negative) at the initiation of treatment.

The decision to discontinue NAs was made by individual physicians using similar, but not uniform, conditions. Four patients who halted NAs for financial reasons were included. No patient underwent interferon treatment during or after NA treatment. The decision to recommence NA administration was also made by individual physicians, essentially when relapse of hepatitis became obvious. With few exceptions, patients were seen at least once a month during the first year after discontinuation of NAs, and at least once every several months afterwards. Stored serum samples were kept frozen at −20°C or below until assayed. This study was approved by the Ethics Committees of all participating institutions.

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and antibody to HBe (anti-HBe) were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd, Tokyo, Japan; Fujirebio Inc., Tokyo, Japan; and/or Sysmex Co., Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[19] was done using a chemiluminescence enzyme immunoassay

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(CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of −1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum concentration of HBV DNA was determined using an Amplicor HBV monitor kit (Roche, Tokyo, Japan),[20] which had a quantitative range of 2.6 to 7.6 log copies/mL. Serum HBV DNA was also determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 9.0 log copies/mL in 43 patients whose serum samples were available at the time of NA discontinuation. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was described as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[22]

Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc., Tokyo, Japan) as described previously.[23,24] Briefly, 150 μL of serum was incubated with pretreatment solution and then added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with a monoclonal antibody mixture against denatured HBcAg, HBeAg, and the 22 kDa precore protein. After incubation and washing, further incubation was carried out with alkaline phosphatase conjugated with two kinds of monoclonal antibodies against denatured HBcAg, HBeAg, and the 22 kDa precore protein. Following washing, a substrate solution was added to the test cartridge and then incubated. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0 to 6.8 log U/mL.

Statistical analyses

A linear regression model was used to examine for associations between mean and maximal values of both ALT and HBV DNA. Correlations between variables were calculated using the Spearman's rank correction correlation coefficient test. Each cut-off value was decided using receiver operating characteristic curve (ROC) analysis and results were evaluated by measuring the area under the curve (AUC). The Fisher's exact and Pearson's χ^2 tests were adopted to test for differences between subgroups of patients. To compare continuous data, the Mann–Whitney *U*-test was used. The Kaplan–Meier method was used to estimate rates of non-relapse observations, and the log-rank test was used to test hypotheses concerning differences in non-relapse observations between selected groups. Multivariate analyses were performed using the Cox regression model. Variables associated with a *P*-value < 0.2 in univariate analyses were included in a stepwise Cox regression analysis to identify independent factors associated with relapse of hepatitis after discontinuation of NAs. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

Definition of hepatitis relapse after discontinuation of NAs

THE CLINICAL CONDITIONS of a successful discontinuation of NAs were set at serum HBV DNA below 4.0 log copies/mL and ALT below 30 IU/L according to the Japanese guidelines for the treatment of hepatitis B.[18] However, these criteria could not be directly applied to our cohort as post-therapy fluctuations in ALT and HBV DNA were difficult to evaluate consistently. In total, 26 (76%) of 34 patients with successful discontinuation of NAs showed transient abnormal levels of ALT and/or HBV DNA, especially during the early phase after cessation. We therefore used mean and maximal values of these markers to evaluate relapse of hepatitis B in this study; mean values were used to evaluate relapse of hepatitis as a whole, and maximal values were used to dynamically assess relapse during the follow-up period after NA discontinuation. Both ALT and HBV DNA were measured 11.0 times per year on average during the first year and 4.1 times per year on average thereafter.

The mean values of HBV DNA were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.853. Similarly, the mean values of ALT were significantly ($P < 0.001$) correlated with maximal values with a correlation coefficient of 0.940 (Fig. 1). The mean HBV DNA value of 4.0 log copies/mL corresponded to a maximal HBV DNA value of 5.7 by ROC analysis (AUC = 0.930, $P < 0.001$), and the mean ALT value of 30 IU/L corresponded to a maximal ALT value of 79 IU/L (AUC = 0.988, $P < 0.001$). These results suggested that patients having serum HBV DNA higher

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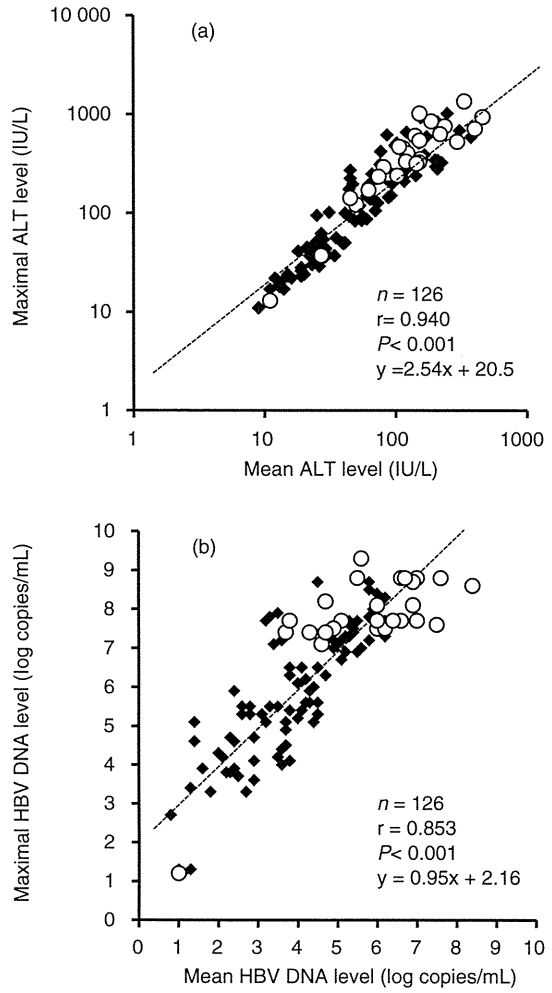

Figure 1 Correlation between maximal and mean levels of alanine aminotransferase (ALT) (a) and hepatitis B virus (HBV) DNA (b) after discontinuation of nucleos(t)ide analogs (NAs). Open circles indicate patients with detectable hepatitis B e antigen (HBeAg) and closed squares indicate patients without detectable HBeAg.

than 5.7 log copies/mL during the follow-up period after NA discontinuation were not likely to achieve the HBV DNA criterion of a successful discontinuation of below 4.0 log copies/mL. Similarly, it could be inferred that patients reaching ALT levels higher than 79 IU/L would also not likely achieve the ALT criterion of a successful discontinuation of below 30 IU/L.

Based on our findings, we judged that a relapse of hepatitis B occurred when serum ALT exceeded 79 IU/L or when serum HBV DNA exceeded 5.7 log copies/mL following NA discontinuation. Accordingly, 92 (73%) of the 126 patients enrolled in the present study showed a relapse. We set the follow-up period as discontinuation to relapse for relapse patients and as discontinuation to the last recorded examination for patients without relapse. Whereas re-administration of NAs due to relapse was commenced in 70% of relapse patients in the follow-up period, none was performed in non-relapse patients during that time.

Elimination of cases likely to show relapse of hepatitis

As it is generally believed that patients who are positive for HBeAg and/or have a higher level of HBV DNA at discontinuation of NAs are likely to relapse, these factors were assessed first. The progression of analyses in the present study and the population structure of each analysis are shown in Figure 2.

The non-relapse rate was compared using the Kaplan–Meier method between 31 patients with HBV DNA equal to or higher than 3.0 log copies/mL and 95 patients with levels lower than 3.0 log copies/mL when NAs were discontinued (Fig. 3). The revised cut-off value of 3.0 log copies/mL was determined by ROC analysis (AUC = 0.709, $P < 0.001$). Thirty (97%) of 31 patients with HBV DNA equal to or higher than 3.0 log copies/mL relapsed within one year of discontinuation. On the other hand, approximately 30% of patients with levels lower than 3.0 log copies/mL showed prolonged non-relapse. Thus, the 31 patients with high HBV DNA at the time of discontinuation were eliminated from the following analyses.

In the remaining 95 patients, the non-relapse rate was compared using the Kaplan–Meier method between 10 patients with detectable HBeAg and 85 patients without HBeAg when NAs were discontinued (Fig. 4). Ninety percent of patients with HBeAg experienced relapse within one year, which was significantly ($P = 0.005$) higher than in cases without HBeAg. In patients without HBeAg, the non-relapse rate decreased rapidly during the first year to approximately 45%, and then decreased relatively slowly over the following 3 years to nearly 30%. It is noteworthy that this subgroup did not relapse afterwards. Since the relapse rate was high among patients with detectable HBeAg, they were excluded from the following analyses as well.

Factors associated with relapse of hepatitis after discontinuation of NAs

Additional factors associated with relapse of hepatitis were analyzed in the remaining 85 patients who were

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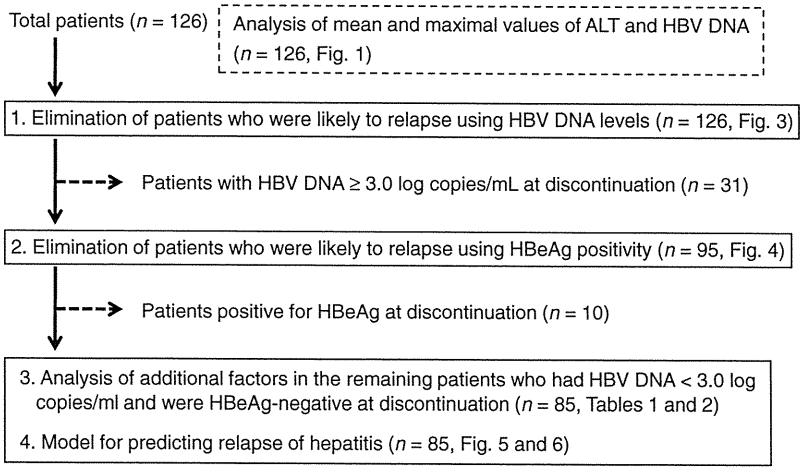

Figure 2 The progression of analyses in the present study and population structure of each analysis.

both negative for HBeAg and whose serum HBV DNA was lower than 3.0 log copies/mL at NA cessation. Table 1 shows the comparison of clinical and virological backgrounds between the 53 relapse and 32 non-relapse patients using univariate analysis. Age and gender distributions were similar between the groups. Approximately 75% of the 85 patients had HBV genotype C, but the distribution of genotypes did not differ between the groups. Approximately 90% of patients were being treated with LVD alone at the time of discontinuation, compared with 6% of patients being given ETV. The median duration of NA treatment was about two times longer in patients without relapse. Levels of both HBsAg and HBcrAg were significantly lower in non-relapse patients than in relapse patients at the time of NA discontinuation. The difference between serum HBsAg was also significant at the initiation of NAs, but not that of HBcrAg. As only patients with HBV DNA lower than 3.0 log copies/mL were analyzed, the majority of these cases showed levels below the 2.6 log copies/mL lower detection limit of the Amplicor assay at NA discontinuation. We therefore also tested HBV DNA with a TaqMan assay, which had a higher sensitivity than the Amplicor assay, in 43 patients whose serum samples were available. The prevalence of patients having a negative detection signal did not differ between the two groups. The number of

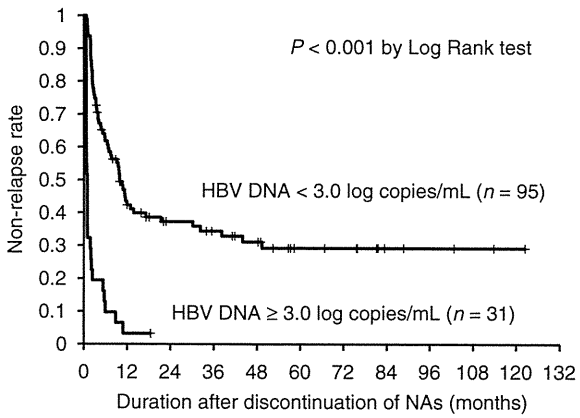

Figure 3 Comparison of non-relapse rates using the Kaplan–Meier method between 31 patients with serum hepatitis B virus (HBV) DNA equal to or higher than 3.0 log copies/mL and 95 patients with serum HBV DNA lower than 3.0 log copies/mL at the time of nucleos(t)ide analog (NA) discontinuation.

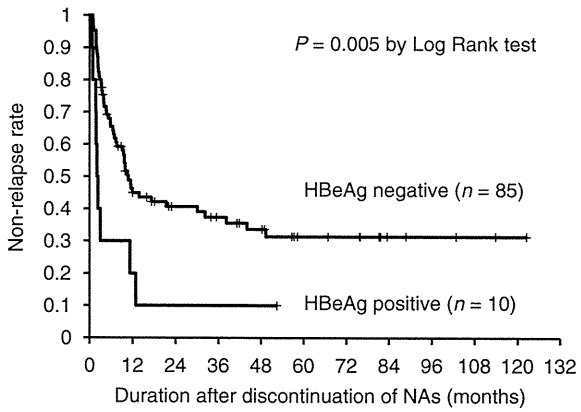

Figure 4 Comparison of non-relapse rates using the Kaplan–Meier method between 10 patients with detectable hepatitis B e antigen (HBeAg) and 85 patients without detectable HBeAg at the time of nucleos(t)ide analog (NA) discontinuation.

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Table 1 Comparison of clinical and virological backgrounds between patients with and without relapse of hepatitis at initiation and discontinuation of nucleos(t)ide analogs (NAs)

Background	Non-relapse patients ($n = 32$)	Relapse patients ($n = 53$)	*P*-value
At initiation of NAs			
Age (years)†	47 (17–75)	48 (26–74)	>0.2
Gender (M : F)	23:9	32:21	>0.2
ALT (IU/L)†	183 (9–1182)	187 (20–2052)	>0.2
Genotype (A : B : C : UD)	1:2:21:8	0:3:44:6	0.193
HBeAg (positive)‡	11 (34%)	16 (30%)	>0.2
HBV DNA			
Amplicor assay (log copies/mL)†	6.2 (<2.6–>7.6)	6.5 (<2.6–>7.6)	0.099
HBsAg (log IU/mL)†	2.7 (0.1–4.3)	3.3 (1.6–3.9)	0.018
HBcrAg (log U/mL)†	5.2 (<3.0–>6.8)	5.6 (<3.0–>6.8)	>0.2
At discontinuation of NAs			
Age (years)†	50 (21–78)	49 (26–79)	>0.2
NAs (LVD : LVD+ADV : ETV : ADV)	28:1:3:0	50:0:2:1	>0.2
Duration of NA treatment (months)†	36 (4–129)	17 (4–84)	0.007
Follow-up period after discontinuation of NAs (months)†	45 (6–123)	12 (1–111)	0.002
ALT (IU/L)†	16 (7–38)	20 (9–65)	0.002
HBV DNA			
Amplicor assay (log copies/mL)†	<2.6 (<2.6–2.9)	<2.6 (<2.6–2.9)	>0.2
TaqMan assay (negative signal)‡	5 (23%) ($n = 22$)	3 (14%) ($n = 21$)	>0.2
TaqMan assay (negative or positive signal)‡	13 (59%) ($n = 22$)	13 (62%) ($n = 21$)	>0.2
HBsAg (log IU/ml)†	2.0 (<–1.5–4.3)	3.1 (0.6–4.0)	0.001
HBcrAg (log IU/mL)†	3.4 (<3.0–4.9)	4.3 (<3.0–>6.8)	0.003

†Data are expressed as the median (range)
‡Data are expressed as a positive number (%)
ADV; adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; LVD, lamivudine; UD, undetermined.

patients with a negative detection signal or a positive signal also did not vary significantly. The follow-up period after discontinuation of NAs was significantly shorter in patients with relapse than in those without because formal follow-up ended once patients relapsed. The median period of follow-up was 45 months in patients without relapse.

Multivariate analyses revealed that a shorter duration of NA treatment and higher levels of HBsAg and HBcrAg at discontinuation were significantly associated with the occurrence of hepatitis relapse (Table 2). The cut-off values that showed the highest significance by ROC analysis were 1.9 log IU/mL for HBsAg (AUC = 0.707, $P = 0.001$), 4.0 log U/mL for HBcrAg (AUC = 0.692, $P = 0.003$), and 16 months (AUC = 0.674, $P = 0.007$) for treatment duration.

Model for predicting relapse of hepatitis using levels of HBsAg and HBcrAg

The existence of a second cut-off value was suggested by ROC analysis for both of HBsAg (2.9 log IU/mL) and HBcrAg (3.0 log IU/mL) to discriminate between

Table 2 Multivariate analysis of factors associated with relapse of hepatitis after discontinuation of nucleos(t)ide analogs (NAs)

Factor	Hazard ratio	95%CI	*P*-value
HBsAg at discontinuation ≥ 1.9 log IU/mL	5.21	1.87–14.55	0.002
HBcrAg at discontinuation ≥ 4.0 log U/mL	2.20	1.25–3.87	0.006
Duration of NA treatment ≥ 16 months	0.54	0.31–0.93	0.027

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen.

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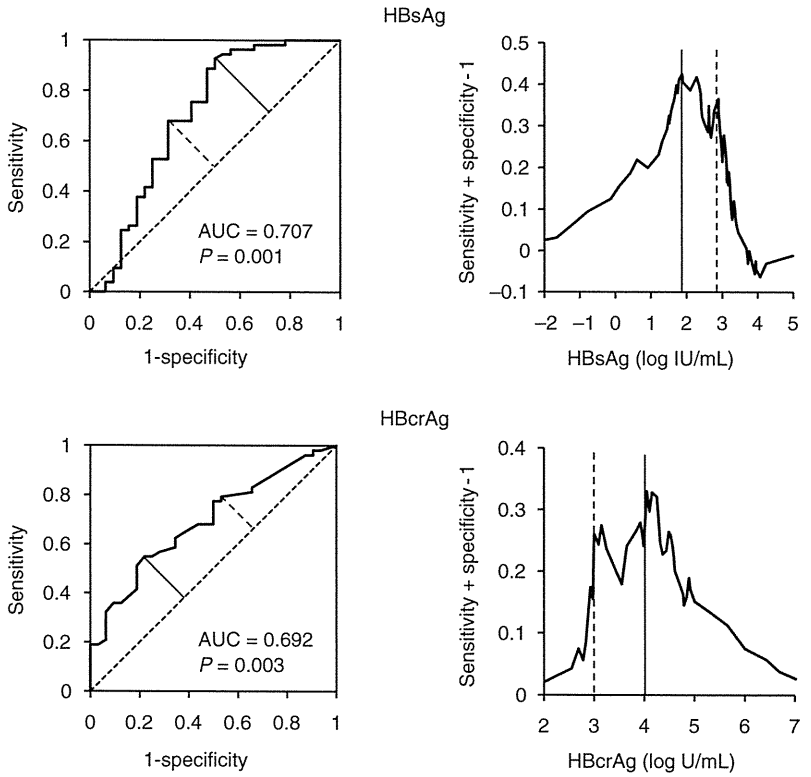

Figure 5 Receiver operating characteristic curve (ROC) analysis of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) to discriminate between patients with and without hepatitis relapse. The existence of two inflection points is suggested for both HBsAg and HBcrAg. Short diagonal lines indicate main inflection points and short broken diagonal lines indicate second inflection points. Vertical lines indicate actual values of antigens that correspond to the main inflection points and vertical broken lines indicate actual values of antigens that correspond to the second inflection points.

patients with and without relapse (Fig. 5). Thus, we set cut-off values as 1.9 and 2.9 log IU/mL for HBsAg and 3.0 and 4.0 log U/mL for HBcrAg in our model for predicting hepatitis relapse.

We tentatively defined three groups using the sum of the scores for HBsAg and HBcrAg levels at the time of NA discontinuation for our model. Conversions were made by assigning a score of 0 for an HBsAg level lower than 1.9 log IU/mL, 1 for a level from 1.9 to 2.8 log IU/mL, and 2 for a level equal to or higher than 2.9 log IU/mL. HBcrAg was scored as 0 for a level lower than 3.0 log U/mL, 1 for a level from 3.0 to 3.9 log U/mL, and 2 for a level equal to or higher than 4.0 log U/mL. Overall, group 1 consisted of patients with a total score of 0, group 2 of patients with a total score of 1 or 2, and group 3 of patients with a total score of 3 or 4.

Patients whose HBV DNA was lower than 3.0 log copies/mL and in whom HBeAg was negative at the time of NA discontinuation were assigned to one of the three groups. Figure 6 shows the comparison of non-relapse rates among the three groups using Kaplan–Meier analysis, which differed significantly. The non-relapse rate was approximately 90% in group 1, as low as 10% in group 3, and intermediate in group 2. When factors associated with relapse were analyzed in group 3 patients, an age of over 40 years at the time of discontinuation was calculated as a significant factor (hazard

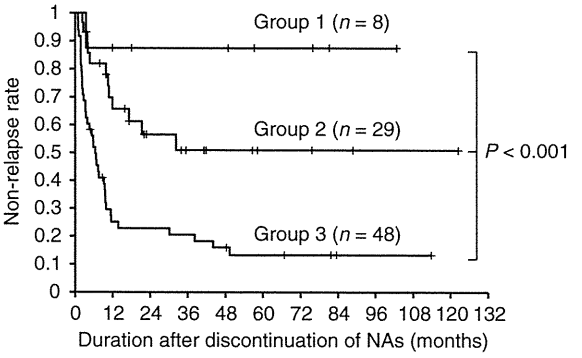

Figure 6 Comparison of non-relapse rates using the Kaplan–Meier method among three groups classified by the sum of the scores of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels at the time of nucleos(t)ide analog (NA) discontinuation.

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ratio = 5.25, range 2.37–11.65, $P < 0.001$). No significant factors were associated with relapse in group 2 patients.

DISCUSSION

THE EUROPEAN ASSOCIATION for the Study of the Liver recommends continuation of NA treatment until HBsAg is cleared.[25] Liu *et al.* came to a similar conclusion in their study of chronic hepatitis B patients treated with LVD.[14] Indeed, the clearance of HBsAg is a reliable marker for the safe discontinuation of NAs, but the rate of patients who can clear HBsAg is relatively low (1–3%/year).[26–28] Thus, additional factors associated with relapse of hepatitis B after discontinuation of NAs were analyzed in the present study to better identify candidates who could achieve drug-free status. Such studies are relatively few, possibly because patients who discontinue NAs prematurely often experience severe complicating relapse and hepatic failure.[9] Although prospective studies are desirable to obtain accurate results, retrospective studies, such as ours, are also necessary to minimize the risk of adverse complications.

Since HBV cannot be completely eradicated in hosts, the primary goal in treating chronic hepatitis B is to convert symptomatic patients into inactive carriers in whom HBeAg is negative (usually anti-HBe-positive), serum HBV DNA is low, and serum ALT is normal.[1,2,18,29] Thus, we set the clinical conditions of a successful discontinuation of NAs as serum HBV DNA level below 4.0 log copies/mL and ALT below 30 IU/L following NA cessation. Patients who satisfy these conditions are not recommended for treatment by the Japanese guidelines for hepatitis B,[18] and it is also widely accepted that the risk of developing cirrhosis or complicating hepatocellular carcinoma is very low in such patients.[30,31] We used our cohort's mean and maximal values of HBV DNA and ALT for relapse analyses. Mean values were useful for evaluating relapse of hepatitis as a whole since parameter levels often fluctuated after discontinuation, and maximal values were used to evaluate relapse in a real-time fashion during the follow-up period. It is noteworthy that the mean and maximal values correlated very closely for both HBV DNA and ALT. The mean HBV DNA value of 4.0 log copies/mL corresponded to the maximal HBV DNA value of 5.7 by ROC analysis, and similarly the mean ALT value of 30 IU/L corresponded to the maximal ALT value of 79 IU/L. Thus, relapse of hepatitis B was judged to occur when serum ALT became higher than 79 IU/L or when serum HBV DNA surpassed 5.7 log copies/mL after the time of NA discontinuation. Such criteria may also be useful for physicians to detect relapse at an early phase and avoid the occurrence of severe reactivation or unnecessary discontinuation of NAs.

It is generally understood that patients with a higher level of HBV DNA at the time of NA discontinuation are likely to relapse, but this cut-off value has not been analyzed sufficiently. Our findings using ROC analysis showed that patients with levels lower than 3.0 log copies/mL have a good possibility to achieve successful discontinuation. The presence of HBeAg is also generally accepted as a reliable factor to predict relapse of hepatitis. Our study showed that patients with detectable HBeAg at the time of NA discontinuation were likely to relapse, even if their HBV DNA levels were lower than 3.0 log copies/mL. Therefore, we next analyzed additional factors associated with a relapse of hepatitis after discontinuation of NAs by selecting patients who met both of these criteria.

Nucleos(t)ide analog treatment produces a rapid decrease in serum HBV DNA by suppressing reverse transcription of pregenomic HBV RNA. However, the key intrahepatic HBV replicative intermediate, covalently closed circular DNA (cccDNA), tends to remain and is capable of reinitiating replication once NAs are ceased.[32] Measurement of HBV cccDNA has been reported to be useful for monitoring and predicting responses to antiviral treatments.[33] However, its measurement is difficult in the clinical setting as it requires a liver biopsy. Due to the mechanism of action of NAs mentioned above, serum HBV DNA does not reflect intrahepatic HBV cccDNA in patients undergoing NA treatment.[34] To address this, quantitative measurement of HBV antigens has been reported to be useful for predicting the effect of antiviral treatment in patients with chronic hepatitis B. Although HBsAg is usually used as a serum marker for the diagnosis of HBV infection, several groups have shown that HBsAg levels can also be reflective of the response to peg-interferon in chronic hepatitis B.[28,35,36] The HBcrAg assay measures serum levels of HB core and e antigens simultaneously using monoclonal antibodies that recognize the common epitopes of these two denatured antigens. Since the assay measures all antigens transcribed from the pre-core/core gene, it is regarded as core-related.[37] Serum HBcrAg has been reported to accurately reflect intracellular levels of HBV cccDNA even during NA treatment,[24,34,38] and was found to be useful for identifying patients who were likely to show relapse of hepatitis after the discontinuation of NAs.[39,40] It is possible that levels of HBsAg and HBcrAg have different roles in

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monitoring antiviral effects because the transcription of these two antigens are regulated by alternative enhancer-promoter systems in the HBV genome.[3] Therefore, we analyzed both of these antigens to elucidate their ability to predict relapse of hepatitis after discontinuation of NAs.

Multivariate analysis demonstrated that levels of HBsAg and HBcrAg at the time of NA discontinuation were independent factors significantly associated with relapse of hepatitis. Thus, we believe these factors can also be applied for predicting relapse in patients whose HBV DNA is lower than 3.0 log copies/mL and whose HBeAg is negative at NA discontinuation. HBV DNA levels were further analyzed using a highly sensitive assay based on real-time polymerase chain reaction (PCR). However, even the level of a negative signal did not ensure successful discontinuation of NAs. The results obtained here indicate that the combined use of HBV-related antigens are useful makers for monitoring the effect of anti-viral treatment in ways different from HBV DNA. Finally, since prolonged NA administration was also a significant factor associated with safe discontinuation, physicians are advised to continue patient treatment for at least 16 months for the best possible outcome.

From our data, a tentative model for predicting relapse of hepatitis after discontinuation of NAs was constructed using levels of HBsAg and HBcrAg at discontinuation. A negative result for HBeAg and HBV DNA lower than 3.0 log copies/mL at the time of NA discontinuation are the essential conditions in this system. Levels of HBsAg and HBcrAg were each converted into scores from 0 to 2 partly because two cut-off values were needed for each antigen and partly because a scoring system may be more convenient for clinical use. The sum of the two scores, which ranged from 0 to 4, was used to prospect relapse. We found that group 1 patients who had a low score (0) could be recommended to discontinue NAs because nearly 90% of this group achieved successful discontinuation. Further analysis of factors associated with relapse are needed for group 2 patients who had middle range scores (1 or 2), since the odds of achieving successful discontinuation were approximately 50%. Continuation of NA treatment is recommended for group 3 patients having high scores (3 or 4) because nearly 90% of this group relapsed. However, this recommendation may be reconsidered in patients younger than 40 years; such cases tended to have a lower relapse rate in group 3. It is also noteworthy that relapse occurred mainly during the first and second years following NA discontinuation in all groups, similarly to a report by Liu *et al.*[14] Thus, clinicians should be vigilant in the early phase after discontinuation.

This study has several limitations. The patients who discontinued NAs were recruited retrospectively, and thus the decision to halt NA treatment was made by individual physicians without uniformly established criteria. Based on this, prospective studies are required to confirm our results. Furthermore, as over 90% of the patients we enrolled had genotype C and over 90% of cases were treated with LVD until discontinuation, the results obtained here can not be applied directly to other HBV genotypes or other types of NAs.

In conclusion, the present study showed that maximal levels of serum ALT and HBV DNA were useful for defining relapse patients after discontinuation of NAs. Along with serum HBV DNA of less than 3.0 log copies/mL and negative serum HBeAg, serum levels of HBsAg and HBcrAg at the time of NA discontinuation were able to predict relapse of hepatitis B and should therefore be considered when establishing uniform guidelines regarding the safe withdrawal of NA treatment. To this end, NA administration of more than 16 months is advisable to achieve successful discontinuation.

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Genome-wide association study identified *ITPA/DDRGK1* variants reflecting thrombocytopenia in pegylated interferon and ribavirin therapy for chronic hepatitis C

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Hematologic abnormalities during current therapy with pegylated interferon and ribavirin (PEG-IFN/RBV) for chronic hepatitis C (CHC) often necessitate dose reduction and premature withdrawal from therapy. The aim of this study was to identify host factors associated with IFN-induced thrombocytopenia by genome-wide association study (GWAS). In the GWAS stage using 900K single-nucleotide polymorphism (SNP) microarrays, 303 Japanese CHC patients treated with PEG-IFN/RBV therapy were genotyped. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the minor-allele-dominant model with the decrease of platelet counts in response to PEG-IFN/RBV therapy [$P = 8.17 \times 10^{-9}$; odds ratio (OR) = 4.6]. These associations were replicated in another sample set ($n = 391$) and the combined *P*-values reached 5.29×10^{-17} (OR = 4.5). Fine mapping with 22 SNPs around *DDRGK1* and *ITPA* genes showed that rs11697186 at the GWAS stage had a strong linkage disequilibrium with rs1127354, known as a functional variant in the *ITPA* gene. The

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ITPA-AA/CA genotype was independently associated with a higher degree of reduction in platelet counts at week 4 ($P < 0.0001$), as well as protection against the reduction in hemoglobin, whereas the CC genotype had significantly less reduction in the mean platelet counts compared with the AA/CA genotype ($P < 0.0001$ for weeks 2, 4, 8, 12), due to a reactive increase of the platelet count through weeks 1–4. Our present results may provide a valuable pharmacogenetic diagnostic tool for tailoring PEG-IFN/RBV dosing to minimize drug-induced adverse events.

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) presents a significant health problem worldwide, with ~2.3% of the world population, i.e. more than 120–130 million people, being infected (1). Only 20–30% of HCV-infected individuals recover spontaneously. The remaining 70–80% go on to develop chronic infection, being at significant risk for progressive liver fibrosis and subsequent liver cirrhosis (LC) and hepatocellular carcinomas (HCC). Successful treatment of chronic hepatitis C (CHC) leads to a reduction of liver fibrosis stage of patients, and also prevents HCC development (2).

Antiviral treatment has been shown to improve liver histology and decrease incidence of hepatocellular carcinoma in CHC (3,4). Current therapy for CHC consists of treatment with pegylated interferon (IFN), which acts both as an antiviral and as an immunoregulatory cytokine, and ribavirin (RBV), an antiviral pro-drug that interferes with RNA metabolism (5,6). However, <50% of patients infected with HCV genotype 1 treated in this way achieve a sustained viral response (SVR) or cure of the infection (5,7). Older patients with liver fibrosis showed a significantly lower SVR rate due to poor adherence resulting from adverse events and laboratory abnormalities (8–10). In particular, hematologic abnormalities often necessitate dose reduction, and premature withdrawal from therapy in 10–14% of patients (5,11–14). New drugs and therapeutic approaches for CHC are actively developed and several candidates are in early trial phase (15,16). Given this background, effective pre-treatment screening for predictive biomarkers with the aim of evaluating possible risks over benefits of currently available treatment will avoid these side effects in patients who will not be helped by treatment, as well as reduce the substantial cost of treatment.

The completion of the Human Genome Project has led to the advent of a new era of scientific research, including a revolutionary approach: the genome-wide association study (GWAS). Several recent studies, including our study, have demonstrated marked associations between single-nucleotide polymorphisms (SNPs) within and around *IL28B* gene, which codes for IFN-λ3 (16–21). Another recent study indicated that genetic variants of *ITPA* gene leading to inosine triphosphatase (ITPA) deficiency could protect against hemolytic anemia (HA) in CHC patients receiving RBV (22).

In Japan, HCV-infected patients are relatively old and some of them have had severe fibrosis (9). Thrombocytopenia is one of the critical adverse events by IFN-based therapy among liver cirrhotic patients (23), because low platelet count (PLT), i.e. <30.0 (10^9/l), would be a risk factor for any bleeding, as well as it would lead to poor treatment efficiency due to the initial or early dose reduction of PEG-IFN. Based on its pathogenesis, drug-induced thrombocytopenia is usually due to bone marrow suppression, immune-mediated destruction and platelet aggregation (24). In this study, we firstly found that genetic variants in the *ITPA/DDRGK1* genes were associated with IFN-induced thrombocytopenia, and then examined the correlation between IFN-induced thrombocytopenia and RBV-induced HA in Japanese CHC patients under PEG-IFN/RBV treatment.

RESULTS

Genetic variants associated with IFN-induced thrombocytopenia

In this study, we conducted a GWAS to identify host genes associated with the decrease of platelets in response to PEG-IFN/RBV treatment in 303 Japanese HCV patients (107 patients with the decrease of PLT versus 196 patients without the decrease of PLT based on the criteria described in Materials and Methods), using a genome-wide SNP typing array (Affymetrix SNP 6.0 for 900K SNPs). The characteristics of patients for each GWAS stage and replication stage are summarized in Table 1. Figure 1 shows a genome-wide view of the single-point association data based on allele frequencies. One SNP (rs11697186) located on *DDRGK1* gene on chromosome 20 showed strong associations in the allele frequency model ($P = 8.17 \times 10^{-9}$) with the decrease of PLT in response to PEG-IFN plus RBV treatment. The association reached genome-wide level of significance [Bonferroni criterion $P < 8.40 \times 10^{-8}$ (0.05/595052)], and another SNP (rs6139030) near *ITPA* gene had a marginal significance ($P = 4.30 \times 10^{-7}$, in Table 2).

To validate the results of the GWAS stage, 22 SNPs were selected for the replication in a set of 391 Japanese HCV patients with and without platelet reduction (Supplementary Material, Table S1). The associations of the original significant SNP (rs11697186) and the marginal SNP (rs6139030) at the GWAS stage were replicated in the second set of 391 patients in the minor-allele-dominant model [$P = 5.88 \times 10^{-10}$, odds ratio (OR) = 4.6 for rs11697186; $P = 3.83 \times 10^{-10}$, OR = 4.3 for rs6139030, Table 2]. The combined P-values for both stages reached 5.29×10^{-17} (OR = 4.5; 95% CI = 3.1–6.5) and 1.33×10^{-15} (OR = 3.9; 95% CI = 2.8–5.5), respectively (Table 2).

Genetic variants associated with RBV-induced anemia

We also conducted a GWAS to identify host genes associated with a quantitative change in hemoglobin (Hb) levels from baseline to week 4 of PEG-IFN/RBV treatment in the above 303 Japanese HCV patients (94 patients with an Hb reduction of ≥3 g/dl at week 4 and 209 patients without Hb reduction), using a genome-wide SNP typing array (Affymetrix SNP 6.0 for 900K SNPs). Two SNPs (rs11697186 and rs6139030)

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