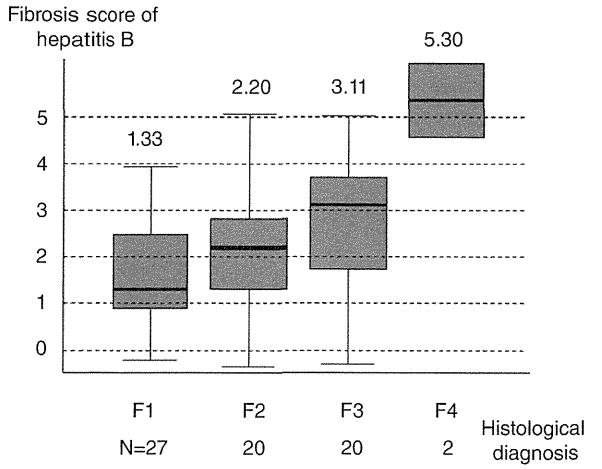

Figure 3 Box and whisker plots of fibrotic score of each group of histological fibrosis in the validation dataset. The fibrosis score of hepatitis B was generated by the function, $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) − $0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) + $1.24 \times ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) + $1.19 \times ln$ (α-2-macroglobulin) (mg/dL) − 9.15.

As many as 227 patients with chronic hepatitis B were analyzed in this study, who had been diagnosed as having chronic hepatitis or cirrhosis by liver biopsy performed in experienced liver units in Japan. To obtain the most suitable equation approximating histological fibrotic stage, multivariate analysis was performed using two demographic parameters (age and sex) and 21 hematological and biochemical markers with or without logarithmic transformation. They included many kinds of fibrosis markers: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, TIMP-1, TIMP-2, procollagen III peptide and type IV collagen 7S Multiple regression analysis finally generated a first-degree polynomial function consisting of four variables: type IV collagen 7S, platelet count, TIMP-2 and α2-macroglobulin. A constant numeral (−9.15) was finally adjusted in the regression equation in order to obtain fitted figures for a fibrotic stage of F1–F4. From the magnitude of the standardized partial regression coefficient of individual variable in the function, platelet count demonstrated the most potent contribution toward the prediction of liver fibrosis. Type IV collagen 7S and *ln* (TIMP-2) proved to be the second and third distinctive power in the model, respectively.

The FSB was sufficiently fitted to actual fibrotic stages with certain overlapping as is usually found in histological ambiguity judged by pathologists. Because judgment of fibrosis in chronic hepatitis often shows a transitional histological staging, pathological examination cannot always make a clear-cut diagnosis discriminating F1–F4. Considering the limitation of the pathological difficulty in differentiating the four continuous disease entities, the obtained regression function showed satisfactory high accuracy rates in the prediction of liver disease severity. The FSB can provide one or two decimal places (e.g. 3.2 or 3.24) and the utility of the score is possibly higher than the mere histological stage of F1–F4. The reproducibility was confirmed by the remaining 67 patients' data obtained from the other six hospitals. Although the validation data were collected from a different geographic area and different chronological situation, the FSB showed similar results in prediction of histological staging.

The FSB seemed a very useful quantitative marker in evaluating fibrotic severity of hepatitis B patients without invasive procedures and without any specialized ultrasonography or magnetic resonance imaging. The FSB also has an advantage of measurement, in which old blood samples are available for retrospective assessment of varied clinical settings: for example, old sera from 20 years prior to the time of initial liver biopsy, or paired sera before and after long-term antiviral therapy. These kinds of retrospective assessments of fibrotic staging will be valuable in estimating a long-term progression of liver disease, in evaluating efficacy of long-term medication or other medical intervention, or in making a political judgment from the viewpoints of socioeconomic efficacy.

The score can be calculated for any patients with chronic HBV infection. Although this multiple regression model dealt with appropriate logarithmic transformation for non-normal distribution parameters, the regression analysis was based on a linear regression model. Very slight fibrosis can be calculated as less than 1.00, which is commonly found to a slight degree in chronic hepatitis with tiny fibrotic change as F0. Very severe fibrosis might be calculated as more than 4.00, which is an imaginary and nonsense number in the scoring system of fibrosis. The FSB is, however, very useful and valuable in a real clinical setting: estimation of severity of liver fibrosis in an outpatient clinic, evaluation of the natural progression of a patient's fibrosis over 10 years and assessment of a long-term administration of interferon in patients with chronic hepatitis B from the viewpoint of fibrotic change. Recent development of new nucleoside/nucleotide analogs requires evaluation for long-term histological advantage, for aggravation of hepatitis stage during viral and biochemical breakthrough caused by HBV mutation, and even for

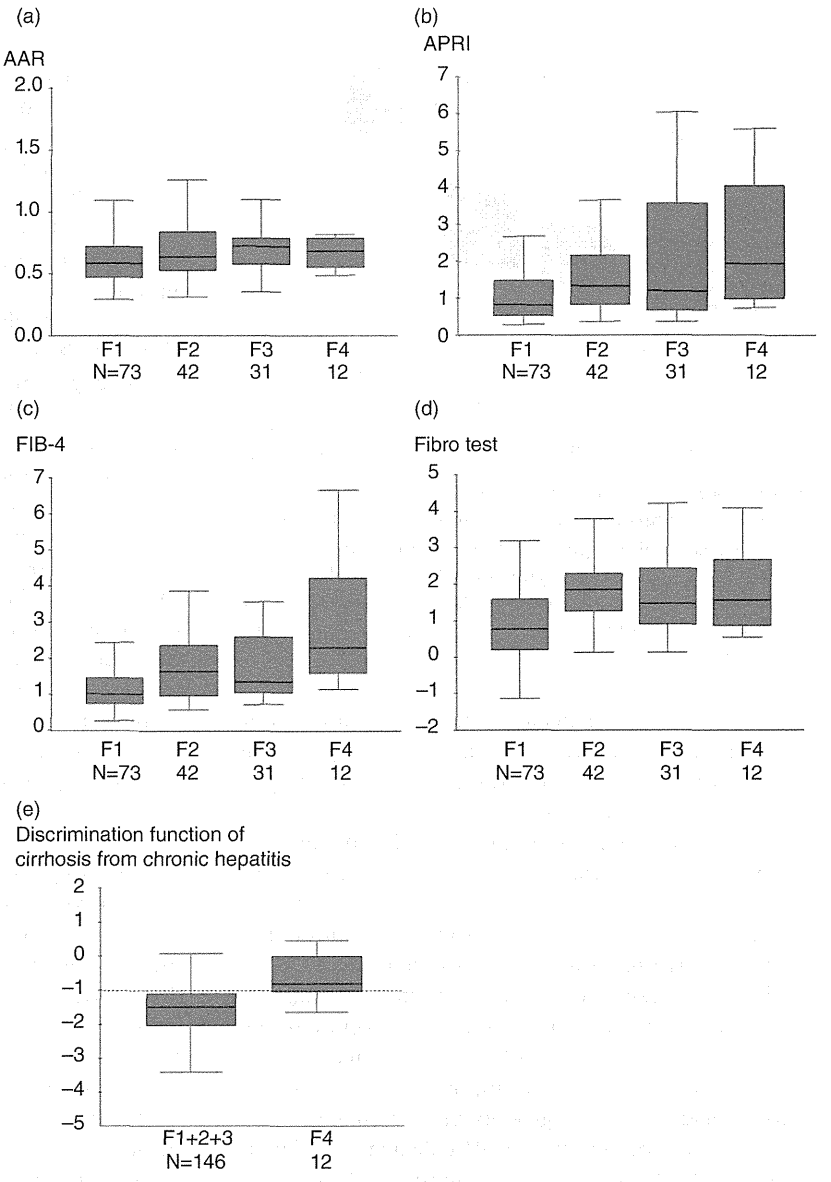

Figure 4 Previously published fibrosis scores. (a) Aspartate aminotransferase/alanine aminotransferase ratio (AAR),[19] (b) aspartate aminotransferase-to-platelet ratio index (APRI),[20] (c) FIB-4,[21] (d) FibroTest[22] and (e) discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

the best management of patients with chronic hepatitis B. The FSB seems one of the ideal methods of approximating the fibrotic stage of chronic hepatitis B. Repeated measurement is quite suitable for patients with an unestablished treatment or trial, every 1 or 2 years, for example. Because the current regression function was generated from the data of HBV-related chronic liver disease, this equation would not be suitable for the recognition of hepatitis C virus-related chronic liver disease, alcoholic liver disease, and other congenital or autoimmune liver diseases. To recognize the latter diseases, other studies of individual diseases must be performed.

We compared the usefulness of the FSB with that of other fibrosis scores.[19–23] The more simple and less expensive AAR or APRI could not estimate fibrotic stages with poor correlation coefficients of 0.199 and 0.265, which are much lower than the coefficient of the FSB of 0.625. FibroTest, which contained three costly fibrosis markers (α-2-macroglobulin, haptoglobin and apolipo-

protein A1), also showed a low correlation coefficient of 0.330, suggesting that its usefulness was limited in HBV positive oriental patients. Although FIB-4 demonstrated the best coefficient of 0.412 among the fibrosis scores, significant overlaps were found between neighboring stages and obtained scores were not coordinated for real histological classification.

In conclusion, the FSB was a useful and reliable biomarker for prediction of liver fibrosis in patients with chronic HBV infection. The FSB is expected to be introduced and utilized in varied kinds of studies and trials. Its accuracy and reproducibility require further validation using higher numbers of patients in several countries other than Japan.

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REFERENCES

1 Sandrin L, Fourquet B, Hasquenoph JM *et al.* Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705–13.
2 Myers RP, Tainturier MH, Ratziu V *et al.* Prediction of liver histological lesions with biochemical markers in patients with chronic hepatitis B. *J Hepatol* 2003; **39**: 222–30.
3 Hanna RF, Kased N, Kwan SW *et al.* Double-contrast MRI for accurate staging of hepatocellular carcinoma in patients with cirrhosis. *AJR Am J Roentgenol* 2008; **190**: 47–57.
4 Hagiwara M, Rusinek H, Lee VS *et al.* Advanced liver fibrosis: diagnosis with 3D whole-liver perfusion MR imaging–initial experience. *Radiology* 2008; **246**: 926–34.
5 Taouli B, Chouli M, Martin AJ, Qayyum A, Coakley FV, Vilgrain V. Chronic hepatitis: role of diffusion-weighted imaging and diffusion tensor imaging for the diagnosis of liver fibrosis and inflammation. *J Magn Reson Imaging* 2008; **28**: 89–95.
6 Montazeri G, Estakhri A, Mohamadnejad M *et al.* Serum hyaluronate as a non-invasive marker of hepatic fibrosis and inflammation in HBeAg-negative chronic hepatitis B. *BMC Gastroenterol* 2005; **5**: 32.
7 Zeng MD, Lu LG, Mao YM *et al.* Prediction of significant fibrosis in HBeAg-positive patients with chronic hepatitis B by a noninvasive model. *Hepatology* 2005; **42**: 1437–45.
8 Chrysanthos NV, Papatheodoridis GV, Savvas S *et al.* Aspartate aminotransferase to platelet ratio index for fibrosis evaluation in chronic viral hepatitis. *Eur J Gastroenterol Hepatol* 2006; **18**: 389–96.
9 Kim BK, Kim Y, Park JY *et al.* Validation of FIB-4 and comparison with other simple noninvasive indices for predicting liver fibrosis and cirrhosis in hepatitis B virus-infected patients. *Liver Int* 2010; **30**: 546–53.
10 Wu SD, Wang JY, Li L. Staging of liver fibrosis in chronic hepatitis B patients with a composite predictive model: a comparative study. *World J Gastroenterol* 2010; **16**: 501–7.
11 Sökücü S, Gökçe S, Güllüoğlu M, Aydoğan A, Celtik C, Durmaz O. The role of the non-invasive serum marker FibroTest-ActiTest in the prediction of histological stage of fibrosis and activity in children with naïve chronic hepatitis B infection. *Scand J Infect Dis* 2010; **42**: 699–703.
12 Liu HB, Zhou JP, Zhang Y, Lv XH, Wang W. Prediction on liver fibrosis using different APRI thresholds when patient age is a categorical marker in patients with chronic hepatitis B. *Clin Chim Acta* 2011; **412**: 33–7.
13 Park SH, Kim CH, Kim DJ *et al.* Usefulness of Multiple Biomarkers for the Prediction of Significant Fibrosis in Chronic Hepatitis B. *J Clin Gastroenterol* 2011; **45**: 361–5.
14 Engstrom-Laurent A, Loof L, Nyberg A, Schroder T. Increased serum levels of hyaluronate in liver disease. *Hepatology* 1985; **5**: 638–42.
15 Murawaki Y, Ikuta Y, Koda M, Kawasaki H. Serum type III procollagen peptide, type IV collagen 7S domain, central triple-helix of type IV collagen and tissue inhibitor of metalloproteinases in patients with chronic viral liver disease: relationship to liver histology. *Hepatology* 1994; **20**: 780–7.
16 Fabris C, Falleti E, Federico E, Toniutto P, Pirisi M. A comparison of four serum markers of fibrosis in the diagnosis of cirrhosis. *Ann Clin Biochem* 1997; **34**: 151–5.
17 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Sheuer PJ. Classification of chronic hepatitis: diagnosis, grading, and staging. *Hepatology* 1994; **19**: 1513–20.
18 IBM SPSS Inc. *IBM SPSS for Windows Version 19.0 Manual.* Armonk NY, USA: SPSS Japan Inc., an IBM company, 2009.
19 Sheth SG, Flamm SL, Gordon FD *et al.* AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. *Am J Gastroenterol* 1998; **93**: 44–8.
20 Wai CT, Greenson JK, Fontana RJ *et al.* A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 518–26.
21 Sterling RK, Lissen E, Clumeck N *et al.* Develoment of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006; **43**: 1317–25.
22 Imbert-Bismut F, Ratziu V, Pieroni L *et al.* Biochemical imarkers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069–75.
23 Ikeda K, Saitoh S, Kobayashi M *et al.* Distinction between chronic hepatitis and liver cirrhosis in patients with hepatitis C virus infection. Practical Discriminant function using common laboratory data. *Hepatol Res* 2000; **18**: 252–66.
24 Lok ASF, McMahon BJ. AASLD Practice Guidelines. Chronic hepatitis B: update 2009. *Hepatology* 2009; **50**: 1–36.

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Original Article

Antitumor efficacy of transcatheter arterial chemoembolization with warmed miriplatin in hepatocellular carcinoma

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Aim: Patients with unresectable hepatocellular carcinoma (HCC) often undergo transcatheter arterial chemoembolization (TACE). Miriplatin is a lipophilic cisplatin derivative used in TACE that is effective in HCC. However, the difference in antitumor efficacy between warmed versus room temperature miriplatin is unclear.

Methods: Chemotherapy efficacy was evaluated by dynamic computed tomography 1–3 months after TACE, according to the Modified Response Evaluation Criteria in Solid Tumors. A total of 203 patients with HCC who received TACE with miriplatin for the first time were included in a follow-up study to retrospectively investigate its efficacy and safety. Overall, 45 patients underwent TACE with warmed (40°C) miriplatin and 158 patients received TACE with room temperature miriplatin.

Results: Seventy patients (44.3%) treated with room temperature miriplatin and 32 patients (71.1%) who received warmed miriplatin experienced complete or partial responses. Multivariate analysis identified miriplatin temperature (warmed miriplatin, risk ratio (RR) = 2.26, $P = 0.047$), tumor number (solitary, RR = 3.48, $P = 0.007$), α-fetoprotein (AFP) level (<50 ng/mL, RR = 2.35, $P = 0.012$) and history of TACE (no history, RR = 2.22, $P = 0.041$) as predictors of objective response following TACE with miriplatin, and no serious complications were observed.

Conclusion: Warm temperature, solitary tumors, low AFP level and first TACE are significant and independent predictors of objective response after TACE using miriplatin. These results suggest that warmed miriplatin can be considered as one of the standard treatments for unresectable HCC.

Key words: hepatocellular carcinoma, miriplatin, transcatheter arterial chemoembolization

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common malignant diseases worldwide.[1] In Japan, more than 30 000 people die of HCC each year, and HCC ranks third and fifth in men and women, respectively, as cause of death due to malignant neoplasms.[2] Because resection, liver transplantation and percutaneous ablation (percutaneous ethanol injection and radiofrequency ablation) are applicable in only 30–40% of HCC patients, transcatheter arterial chemoembolization (TACE) has been recognized as an effective palliative treatment option for patients with advanced HCC.[3–10] TACE is recommended for HCC patients with class A or B liver damage, two or three tumors, and a tumor diameter greater than 3 cm, according to the guidelines for treatment of HCC by the Japan Society of Hepatology in 2009.[11] The Barcelona Clinic Liver Cancer group recommends TACE for HCC patients with stage B and class A or B disease and more than four tumors, or stage C disease without portal vein invasion or extrahepatic metastasis.[12] Miriplatin (cis-[1R,2R]-1,2-cyclohexanediamine-N,N']bis[myristate])-platinum(II) monohydrate; Dainippon Sumitomo Pharma, Osaka, Japan) is a novel lipophilic cisplatin derivative that can be suspended in lipiodol, a lipid lymphographic agent.[13–16] Some trials reported that miriplatin is effective for HCC.[17,18] Addition of embolizing agents to miriplatin-based treatment has been shown to result in a higher response in patients with

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HCC.[19] Significant predictors for complete response to miriplatin include solitary tumors, previous complete response to TACE via injection from the peripheral to segmental hepatic artery,[20] and stage I or II disease.[21] The most important issue regarding TACE with miriplatin is its viscosity: due to its high viscosity, miriplatin/lipiodol suspension cannot enter smaller vessels. We previously determined that warming miriplatin to 40°C decreased its viscosity in vitro (unpubl. obs.). We investigated the viscosity of miriplatin/lipiodol suspension using a viscometer (μVISC; RHEOSENSE, San Ramon, CA, USA). The miriplatin/lipiodol suspension was adjusted to 20 mg/mL, and then warmed to 40°C. We measured the viscosity of these solutions at room temperature and 40°C three times, and determined that the mean viscosity of miriplatin/lipiodol suspension at room temperature and 40°C is 37.48 mPa-S and 21.42 mPa-S, respectively. The purpose of this retrospective study was to evaluate the antitumor efficacy and adverse effects of TACE with warmed miriplatin suspension.

METHODS

Patients

A TOTAL OF 402 HCC Japanese adult patients were consecutively recruited into the study protocol of TACE with miriplatin from December 2007 to June 2012 at our center. Among them, 203 patients who received miriplatin for the first time and who were assessed 1–3 months after TACE were enrolled in this retrospective study. Warmed miriplatin was used for all patients from August 2011 to June 2012. Overall, 45 patients received warmed miriplatin and 158 patients received room temperature miriplatin.

Table 1 summarizes the profile and laboratory data of the study patients. The median follow-up period, from the end of TACE until the last visit, was 458 days (range, 57–1226 days). Higher serum aspartate aminotransferase (AST) levels and prothrombin activity were observed in patients in the room temperature miriplatin group compared to those in the warmed miriplatin group. The study protocol was approved by the ethics committee of our hospital, and written informed consent was obtained from all participating patients.

HCC

Before treatment with miriplatin, all patients underwent a comprehensive evaluation consisting of a medical history, physical examination, measurement of tumor size, performance status, chest radiograph, liver-imaging studies (dynamic computed tomography [CT], ultrasonography [US], digital-subtraction angiography [DSA]), complete blood count and blood chemistry. Diagnosis of HCC was established based on the findings

Table 1 Profile and pretreatment laboratory data of 203 patients who underwent TACE using miriplatin/lipiodol suspension under room temperature and warmed conditions for unresectable HCC

	Total	Room temperature miriplatin group	Warmed miriplatin group	*P*-value
Demographic data				
No. of patients	203	158	45	
Sex (male/female)	130/73	99/59	31/14	0.485
Age, years†	73 (45–91)	71 (45–91)	74 (48–86)	0.940
Etiology, HBV/HCV/other	24/161/18	17/130/11	7/31/7	0.097
Laboratory data†				
Albumin, g/dL	3.0 (2.0–4.2)	3.3 (2.0–4.2)	3.0 (2–4.1)	0.553
Serum aspartate aminotransferase, IU/L	50 (18–415)	52 (18–415)	47 (19–305)	0.033
Serum alanine aminotransferase, IU/L	34 (12–282)	34 (12–171)	31 (12–282)	0.311
Total bilirubin, mg/dL	1.0 (0.4–4.9)	1.1 (0.4–4.9)	1.0 (0.4–2.7)	0.902
Platelet count, $\times 10^3/mm^3$	9.6 (1.9–28.2)	9.5 (1.9–28.2)	10.0 (3.5–26.5)	0.716
Prothrombin activity, %	79.2 (40.8–123.1)	81.5 (45.7–123.1)	74.0 (40.8–106.1)	0.005
AFP, μg/L	30.0 (1.8–282 200)	32.3 (1.8–282 200)	22.0 (2.9–49 710)	0.527
AFP-L3, %	19.0 (0–82.7)	22.7 (0–82.7)	12.0 (0–78.0)	0.601
DCP, AU/L	39.0 (4–662 000)	40.5 (4–65 290)	30 (8–662 000)	0.748
Child–Pugh class, A/B	152/51	119/39	33/12	0.846

Data are shown as number and percentage of patients, except those denoted by †, which represent the median (range) values.
AFP, α-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; DCP, des-γ-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; TACE, transcatheter arterial chemoembolization.

of dynamic CT, US and DSA. Patients who had extrahepatic metastasis of HCC or other malignancies were excluded.

Table 2 summarizes the tumor profiles and TACE treatment history of patients in each study group. In the warmed miriplatin group, 12 patients (26.7%) had a solitary tumor and 33 patients (73.3%) had multiple tumors. The median diameter of the largest tumor was 30 mm (range, 6–115 mm) and 29 patients (64.4%) had a history of TACE. In the room temperature miriplatin group, 29 patients (18.4%) had a solitary tumor and 129 patients (81.6%) had multiple tumors. The median diameter of the largest tumor was 30 mm (range, 6–125 mm), and 120 patients (75.9%) had a history of TACE. Patients in the room temperature miriplatin group tended to have more tumors than those in the warmed miriplatin group.

Treatment protocol

Patients were hydrated through a peripheral line. The femoral artery was catheterized under local anesthesia, and a 4-Fr Shepherd Hook catheter (FansacIV or Angiomaster; Terumo Clinical Supply, Gifu, Japan) was inserted into the hepatic artery, and portography through the superior mesenteric artery and celiac arteriography were performed. Then, a 2.0- or 2.1-Fr microcatheter was advanced into the feeding arteries of each tumor, and miriplatin suspended in lipiodol solution was injected into the hepatic artery; however, the injection was discontinued immediately before the flow ceased completely. Thereafter, the feeding arteries to the tumors were embolized with 1-mm gelatin cubes (Gelpart; Nippon Kayaku, Tokyo, Japan). The miriplatin/lipiodol suspension was administrated slowly under careful fluoroscopic guidance. The dose of miriplatin/lipiodol was 120–180 mg/2–3 mL and was determined based on tumor size and degree of liver dysfunction. A 5-HT3 antagonist was administrated before the miriplatin injection; however, hydration by i.v. fluid administration was not conducted before the TACE procedure. A clean container was placed in an electric range filled with water. The injector of miriplatin/lipiodol suspension and sterilized physiological saline were then placed in the container, and the container was warmed to 60°C. We observed that in 60°C water, the miriplatin/lipiodol suspension in the injector reaches 40°C *in vitro*. The stability of warmed miriplatin/lipiodol suspension has been previously reported.

Assessment of therapeutic efficacy

The efficacy of chemotherapy was evaluated by dynamic CT 1–3 months after TACE with miriplatin, and was based on change in the maximum diameter of viable target lesions (i.e. those showing enhancement in the arterial phase). Response categories, according to the Modified Response Evaluation Criteria in Solid Tumors[22] are as follows: complete response (CR), disappearance of any intratumoral arterial enhancement in all target lesions; partial response (PR), at least a 30% decrease in the sum of diameters of viable target lesions; stable disease (SD), any cases that do not qualify for either PR or progressive disease; and progressive disease (PD), an increase of at least 20% in the sum of the diameters of viable target lesions.

Toxicity evaluation

Treatment-related toxicity was assessed using the National Cancer Institute Common Terminology Criteria (ver. 4.0). Within 2 weeks before TACE with miriplatin, and at 3–7 days (three times during this period) and at 1 month afterward, hematological (i.e. leukocyte and thrombocyte counts) and clinical chemistry (i.e. serum AST, serum alanine aminotransferase [ALT],

Table 2 Tumor profile and treatment history of 203 patients who underwent TACE using miriplatin/lipiodol suspension under room temperature condition and warmed conditions for unresectable HCC

	Total	Room temperature miriplatin group	Warmed miriplatin group	*P*-value
No. of patients	203	158	45	
Tumor size, mm†	20 (6–125)	30 (6–125)	30 (6–115)	0.435
Tumor multiplicity (solitary/multiple)	41/162	29/129	12/33	0.291
No. of tumors†	3 (1–100)	3 (1–100)	3 (1–40)	0.030
Stage (I/II/III/IV)	54/81/66/2	38/67/51/2	16/14/15/0	0.329
History of TACE	73.4%	75.9%	64.4%	0.130

Data are shown as number and percentage of patients, except those denoted by †, which represent the median (range) values.
HCC, hepatocellular carcinoma; TACE, transcatheter arterial chemoembolization.

albumin, total bilirubin, serum creatine and prothrombin activity) toxicity evaluations were conducted.

Statistical analysis

The distribution of subject characteristics was assessed by the χ^2-test or Mann–Whitney *U*-test, as appropriate. Logistic analysis was used to determine independent predictive factors associated with CR and PR by TACE with miriplatin. The risk ratio (RR) and 95% confidence interval (CI) were also calculated. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into a multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using SPSS software (SPSS, Chicago, IL, USA). All *P*-values of less than 0.05 by two-tailed test were considered significant.

RESULTS

Treatment effects

OF THE 203 treated patients, 55 (27.1%) experienced a CR, 47 patients (23.2%) PR, 66 patients (32.5%) SD and 33 patients (17.2%) PD. Overall, 50.3% of patients achieved an objective response (i.e. CR plus PR).

Predictive factors associated with objective response to TACE

Data from the entire study population were analyzed to identify factors that could predict objective response. Univariate analysis identified five parameters that tended to correlate or significantly correlated with objective response: miriplatin temperature (warmed miriplatin, $P = 0.002$), tumor number (solitary tumor, $P < 0.001$), α-fetoprotein (AFP) level (<50 ng/mL, $P = 0.003$), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%) (<10%, $P = 0.032$) and history of TACE (no history, $P = 0.002$). These five factors were entered into multivariate analysis, which revealed four parameters to be significant and independent determinants of objective response using miriplatin: miriplatin temperature (warmed miriplatin, risk ratio [RR] = 2.26, $P = 0.047$), tumor number (solitary tumor, RR = 3.48, $P = 0.007$), AFP level (<50 ng/mL, RR = 2.35, $P = 0.012$) and history of TACE (no history, RR = 2.22, $P = 0.041$) (Table 3).

Objective response according to AFP-L3%

Patients were divided into two groups according to AFP-L3 serum level using a cut-off value of 10% (low AFP-L3 group [<10%], $n = 83$; high AFP-L3 group [≥10%], $n = 89$). In the high AFP-L3 group, 27 of 83 patients (32.5%) experienced CR, 22 patients (26.5%) PR, 26 patients (31.3%) SD and eight patients (9.6%) PD. In the low AFP-L3 group, 17 of 89 patients (19.1%) experienced CR, 20 patients (22.5%) PR, 29 patients (32.6%) SD and 23 patients (25.8%) PD. The response rates were significantly different between the two groups ($P = 0.032$, log-rank test).

Objective response according to miriplatin temperature, tumor number, AFP and history of TACE

Next, the efficacy of TACE using miriplatin according to temperature condition was examined (Fig. 1). In the warmed miriplatin group, 19 of 45 patients (42.2%) experienced CR, 13 patients (28.9%) PR, eight patients (17.8%) SD and five patients (11.1%) PD. In the room temperature miriplatin group, 36 of 158 patients (22.8%) experienced CR, 34 patients (21.5%) PR, 58

Table 3 Factors associated with objective response (CR plus PR) after TACE using miriplatin, identified by multivariate analysis

Factors	Category	Risk ratio (95% confidence interval)	*P*-value†
Miriplatin condition	1: Room temperature	1	0.047
	2: Warmed	2.26 (1.01–5.04)	
Tumor number	1: Multiple nodules	1	0.007
	2: Solitary nodule	3.48 (1.42–8.62)	
AFP	1: ≥50 ng/mL	1	0.012
	2: <50 ng/mL	2.35 (1.21–4.57)	
History of TACE	1: Yes	1	0.041
	2: No	2.22 (1.03–4.75)	

†Cox proportional hazard model.
AFP, α-fetoprotein; CR, complete response; PR, partial response; TACE, transcatheter arterial chemoembolization. [Correction made after online publication on 14 March 2013: Category 1 of AFP was changed to ≥50 ng/mL, and category 2 of AFP was changed to <50ng/mL.]

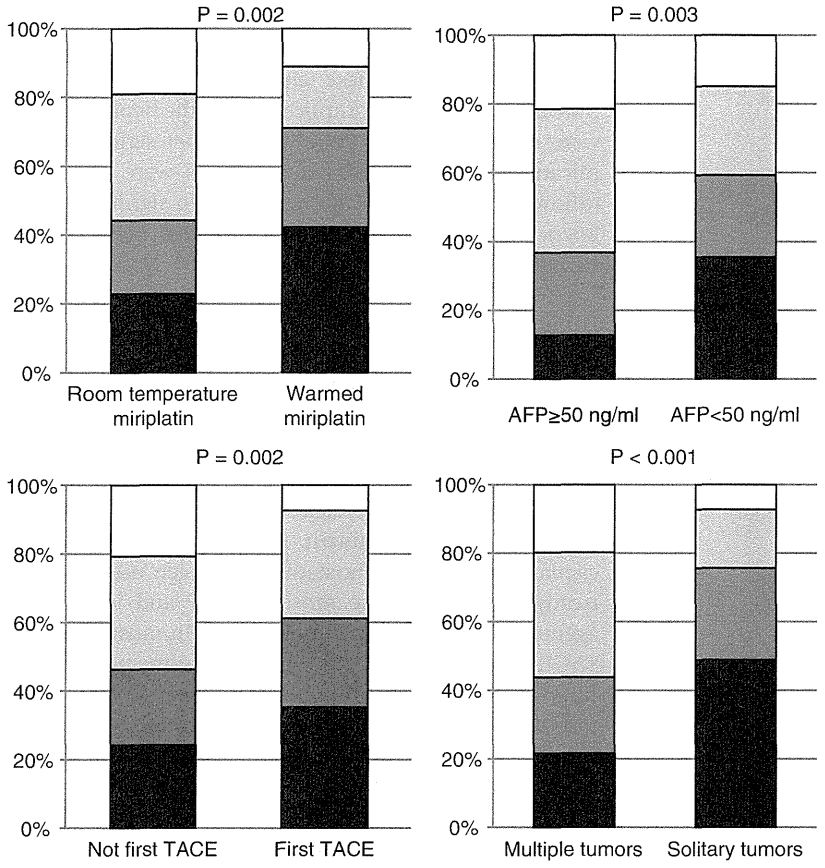

Figure 1 Efficacy of transcatheter arterial chemoembolization (TACE) using miriplatin in patients with hepatocellular carcinoma according to miriplatin temperature, serum α-fetoprotein (AFP) level, history of TACE and tumor number. Complete response (CR) and partial response (PR) rates were significantly higher for patients who received warmed miriplatin, had a low AFP level, were undergoing their first TACE and/or had solitary tumors. □, progressive disease (PD); ▨, stable disease (SD); ▩, PR; ■, CR. [Correction made after online publication on 14 March 2013: In the history of TACE diagram, the left column was relabeled as 'Not first TACE' while the right column was relabeled as 'First TACE'.]

patients (36.7%) SD and 30 patients (19.0%) PD. Overall, 71.1% of patients in the warmed miriplatin group and 44.3% of patients in the room temperature miriplatin group experienced an objective response (i.e. CR plus PR). The rates were significantly different between the two groups ($P = 0.002$, log-rank test).

In the high AFP group (≥50 ng/mL, $n = 79$), 10 of 79 patients (12.7%) experienced CR, 19 patients (24.1%) PR, 33 patients (41.8%) SD and 17 patients (21.5%) PD. In the low AFP group (<50 ng/mL, $n = 113$), 40 of 113 patients (35.4%) experienced CR, 27 patients (23.9%) PR, 29 patients (25.7%) SD and 17 patients (15.0%) PD (Fig. 1). The rates were significantly different between the two groups ($P = 0.003$, log-rank test).

In the TACE-naïve group ($n = 54$), 19 of 54 patients (35.2%) experienced CR, 14 patients (25.9%) PR, 17 patients (31.5%) SD and four patients (7.4%) PD. In patients who had previously undergone TACE ($n = 149$), 36 of 149 patients (24.2%) experienced CR, 33 patients (22.1%) PR, 49 patients (32.9%) SD and 31 patients (20.8%) PD (Fig. 1). The rates were significantly different between the two groups ($P = 0.002$, log-rank test).

Among all patients, 41 patients (20.2%) had a solitary tumor and 162 (79.8%) had multiple tumors. In the solitary tumor group, 20 of 41 treated patients (48.8%) experienced CR, 11 patients (26.8%) PR, seven patients (17.1%) SD and three patients (7.3%) PD. In the multiple tumors group, 35 of 162 patients (21.6%) experienced CR, 36 patients (22.2%) PR, 59 patients (36.4%) SD and 32 patients (19.8%) PD (Fig. 1). The rates were significantly different between the two groups ($P < 0.001$, log-rank test).

Adverse effects

Fever, anorexia and elevated serum transaminase levels were observed in most patients after miriplatin administration (Table 4). In the room temperature miriplatin group and warmed miriplatin groups, the following grade 4 events were observed: increased AST in four

Table 4 Adverse effects following miriplatin administration

	Room temperature condition ($n = 158$)				Warmed condition ($n = 45$)			
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4
White blood cells decreased	11 (7.0%)	19 (12.0%)	1 (0.6%)	0	5 (10.7%)	4 (8.9%)	0	0
Anemia	96 (60.8%)	19 (12.0%)	5 (3.2%)	0	21 (46.7%)	6 (17.9%)	0	0
Platelet count decreased	80 (50.6%)	38 (24.1%)	20 (12.7%)	0	22 (48.9%)	10 (22.2%)	3 (6.7%)	0
Aspartate aminotransferase increased	75 (47.5%)	33 (20.9%)	38 (24.1%)	4 (2.5%)	21 (46.7%)	7 (15.6%)	20 (28.6%)	2 (4.4%)
Alanine aminotransferase increased	74 (46.8%)	17 (10.8%)	22 (13.9%)	1 (0.6%)	21 (46.7%)	10 (22.2%)	4 (8.9%)	2 (4.4%)
Fever	72 (45.6%)	17 (10.8%)	0	0	22 (48.9%)	7 (17.9%)	0	0
Appetite loss	63 (39.9%)	2 (1.3%)	0	0	25 (55.6%)	0	0	0
Abdominal pain	30 (0.6%)	5 (3.2%)	0	0	4 (10.7%)	2 (4.4%)	0	0

Values denote numbers of subjects. Treatment-related toxicity was assessed using the National Cancer Institute Common Terminology Criteria ver. 4.0.

(2.5%) and one patient (3.5%), respectively, and increased ALT in one (0.6%) and one patient (3.6%), respectively; all of these elevations resolved within 2 weeks. No vascular complications of the hepatic artery were observed in any patient. No other serious complications or treatment-related deaths were observed following miriplatin administration. No significant differences in adverse effects were observed between the two groups.

DISCUSSION

TRANSCATHETER ARTERIAL CHEMOEMBOLIZATION is widely performed in patients with HCC who are not eligible for curative therapy. Previous randomized controlled trials and meta-analyses confirmed the survival benefit of TACE. Because many anticancer drugs, such as doxorubicin, epirubicin, mitomycin C, cisplatin and neocarzinostatin, have been used for the treatment of HCC, the most effective and least toxic agents or protocol remain unclear.[23,24] In most patients, TACE can be repeated, and using the same agent multiple times can lead to resistance. A previous study reported that platinum analogs are frequently effective for advanced HCC that are unresponsive to TACE with epirubicin.[25] Miriplatin was developed as a lipophilic platinum complex that has superior antitumor efficacy in HCC with lower toxicity compared to cisplatin.[13–16] Previous reports suggested that TACE with miriplatin can be used safely for HCC patients with chronic renal failure.[26]

Pharmacokinetic studies have demonstrated that the plasma concentration of total platinum is much lower in patients treated with miriplatin compared with that in patients treated with intra-arterial cisplatin: the C_{max} is approximately 300-fold lower and the T_{max} roughly 500-fold longer for miriplatin than the corresponding values for intra-arterial cisplatin. Miriplatin/lipiodol suspension is a stable colloidal emulsion that is deposited within HCC tumors, where it gradually releases active derivatives of miriplatin. Miriplatin/lipiodol releases 1,2-diaminocyclohexane platinum (II) dichloride (DPC) as its active platinum compound, which binds to nuclear DNA and mediates miriplatin/lipiodol cytotoxicity. In a cisplatin-resistant rat hepatoma cell line model, cross-resistance to DPC was not observed.[27]

Previous studies reported the efficacy of miriplatin, but differences in efficacy associated with miriplatin temperature have not yet been evaluated. In the present study, we examined predictors of objective response to TACE with miriplatin. Multivariate analysis identified

use of warmed miriplatin, low serum AFP, first TACE and solitary tumors as predictors of objective response in patients who received TACE with miriplatin. Previous reports identified CR after previous TACE, solitary tumor, injection from peripheral to segmental hepatic artery,[20] and stage I or II disease[21] as significant predictors associated with CR to TACE with miriplatin. Another report stated that the rates of local recurrence and intratumoral recurrence in patients treated with epirubicin were significantly lower than those in patients treated with miriplatin.[28] In the present study, some of the above factors were not identified as significant predictors of response. The differences in the findings of the present study and the reports described above are not currently clear, but may reflect differences in the population samples, as this was the first study to focus on the objective response of patients receiving miriplatin for the first time. Notably, the present study is the first study to investigate the viscosity of miriplatin/lipiodol suspension. Further studies of larger populations including individuals of other ethnicities are necessary.

In this study, warmed miriplatin was associated with objective response after TACE. The main issue associated with miriplatin administration is its high viscosity, which prevents the miriplatin/lipiodol suspension from flowing into the peripheral artery and leads to inhomogeneous distribution of miriplatin/lipiodol suspension in HCC tumors. This is the primary reason that TACE with miriplatin is associated with reduced efficacy compared to TACE with other agents.[28] Basic research has provided evidence that as the temperature of miriplatin/lipiodol suspension rises, its viscosity decreases; for example, the viscosity of miriplatin/lipiodol suspension at 40°C is 0.51-times that at 25°C. The chemical behavior of miriplatin does not change until its temperature reaches 70°C. Further studies should be performed to investigate the viscosity and antitumor efficacy of condensed and warmed miriplatin conditions, as well as the associated wash-out periods. In addition, although no significant differences in adverse effects between groups were noted, further follow up regarding vascular complications of the hepatic artery is required.

Previous studies reported the relationship between tumor multiplicity and efficacy of TACE.[20] TACE can be performed selectively, and the dose of drug per tumor is higher in patients with solitary tumors than in those with multiple tumors. In the present study, solitary tumors and warmed miriplatin were associated with objective response. These results are not inconsistent with previous studies. Interestingly, in the present patients, the impact of warmed miriplatin and solitary tumor was more significant than that of age, liver function, tumor size, tumor stage, tumor markers, injection artery and history of TACE. One possible explanation for this finding is that the study population included patients who received TACE with miriplatin for the first time. Previous studies reported that complete tumor necrosis after TACE offered favorable long-term survival outcomes in HCC patients.[5,29] In the current study, warmed miriplatin administration was associated with objective response, suggesting that warmed miriplatin administration potentially results in a favorable prognosis for HCC.

The present study has certain limitations. This was a retrospective study and the patients were not randomized with respect to treatment with warmed versus room temperature miriplatin. A prospective study is needed to assess the safety and efficacy of warmed miriplatin administration. The other limitation is the small number of cases in the warmed miriplatin group. A study with a larger number of patients is required to confirm the present results. Furthermore, evaluation of the efficacy of warmed miriplatin compared with epirubicin or cisplatin in HCC is also required.

In conclusion, the present study identified warmed miriplatin and solitary tumors as significant and independent predictors of objective response after TACE using miriplatin. The results emphasize the importance of the condition under which miriplatin is administrated, and we recommend that warmed miriplatin should be the standard method of administration for patients with unresectable HCC undergoing TACE.

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REFERENCES

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Umemura T, Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; **44** (Suppl): 102–7.
3 Yamada R, Sato M, Kawabata M, Nakatsuka H, Nakamura K, Takashima S. Hepatic artery embolization in 120 patients with unresectable hepatoma. *Radiology* 1983; **148**: 397–401.
4 Lin DY, Liaw YF, Lee TY, Lai CM. Hepatic arterial embolization in patients with unresectable hepatocellular carcinoma: a randomized controlled trial. *Gastroenterology* 1988; **94**: 453–6.

5 Ikeda K, Kumada H, Saitoh S, Arase Y, Chayama K. Effect of repeated transcatheter arterial embolization on the survival time in patients with hepatocellular carcinoma. *Cancer* 1991; **68**: 2150–4.
6 Llovet JM, Real MI, Montana X *et al.*; Barcelona Liver Cancer Group. Arterial embolisation or chemoembolization versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002; **359**: 1734–9.
7 Lo CM, Ngan H, Tso WK *et al.* Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. *Hepatology* 2002; **35**: 1164–71.
8 Camma C, Schepis F, Orlando A *et al.* Transarterial chemoembolization for unresectable hepatocellular carcinoma: meta-analysis of randomized controlled trials. *Radiology* 2002; **224**: 47–54.
9 Ikeda M, Maeda S, Shibata J *et al.* Transcatheter arterial chemotherapy with and without embolization in patients with hepatocellular carcinoma. *Oncology* 2004; **66**: 24–31.
10 Takayasu K, Arii S, Ikai I *et al.*; Liver Cancer Study Group of Japan. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. *Gastroenterology* 2006; **131**: 461–9.
11 Arii S, Sata M, Sakamoto M *et al.* Management of hepatocellular carcinoma: report of consensus meeting in the 45th annual meeting of the Japan Society of Hepatology (2009). *Hepatol Res* 2010; **40**: 667–85.
12 Bruix J, Sala M, Llovet JM. Chemoembolization for hepatocellular carcinoma. *Gastroenterology* 2004; **127** (5 Suppl 1): S179–S188.
13 Maeda M, Uchida NA, Sasaki T. Liposoluble platinum (II) complexes with antitumor activity. *Jpn J Cancer Res* 1986; **77**: 523–5.
14 Kishimoto S, Ohtani A, Fukuda H, Fukushima S, Takeuchi Y. Relation between intracellular accumulation and cytotoxic activity of cis-[((1R,2R)-1, 2-cyclohexanediamine- N, N')bis(myristato)]platinum(II) suspended in Lipiodol. *Biol Pharm Bull* 2003; **26**: 683–6.
15 Hanada M, Baba A, Tsutsumishita Y, Noguchi T, Yamaoka T. Intra-hepatic arterial administration with miriplatin suspended in an oily lymphographic agent inhibits the growth of human hepatoma cells orthotopically implanted in nude rats. *Cancer Sci* 2009; **100**: 189–94.
16 Hanada M, Baba A, Tsutsumishita Y *et al.* Intra-hepatic arterial administration with miriplatin suspended in an oily lymphographic agent inhibits the growth of tumors implanted in rat livers by inducing platinum-DNA adducts to form and massive apoptosis. *Cancer Chemother Pharmacol* 2009; **64**: 473–83.
17 Fujiyama S, Shibata J, Maeda S *et al.* Phase I clinical study of a novel lipophilic platinum complex (SM-11355) in patients with hepatocellular carcinoma refractory to cisplatin/lipiodol. *Br J Cancer* 2003; **89**: 1614–19.
18 Okusaka T, Okada S, Nakanishi T, Fujiyama S, Kubo Y. Phase II trial of intra-arterial chemotherapy using a novel lipophilic platinum derivative (SM-11355) in patients with hepatocellular carcinoma. *Invest New Drugs* 2004; **22**: 169–76.
19 Imai N, Ikeda K, Kawamura Y *et al.* Transcatheter arterial chemotherapy using miriplatin-lipiodol suspension with or without embolization for unresectable hepatocellular carcinoma. *Jpn J Clin Oncol* 2012; **42**: 175–82.
20 Imai N, Ikeda K, Seko Y *et al.* Previous chemoembolization response after transcatheter arterial chemoembolization (TACE) can predict the anti-tumor effect of subsequent TACE with miriplatin in patients with recurrent hepatocellular carcinoma. *Oncology* 2011; **80**: 188–94.
21 Imai Y, Chikayama T, Nakazawa M *et al.* Usefulness of miriplatin as an anticancer agent for transcatheter arterial chemoembolization in patients with unresectable hepatocellular carcinoma. *J Gastroenterol* 2011 [Epub ahead of print].
22 Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis* 2010; **30**: 52–60.
23 Marelli L, Stigliano R, Triantos C *et al.* Transarterial therapy for hepatocellular carcinoma: which technique is more effective? A systematic review of cohort and randomized studies. *Cardiovasc Intervent Radiol* 2007; **30**: 6–25.
24 Kamada K, Nakanishi T, Kitamoto M *et al.* Long-term prognosis of patients undergoing transcatheter arterial chemoembolization for unresectable hepatocellular carcinoma: comparison of cisplatin lipiodol suspension and doxorubicin hydrochloride emulsion. *J Vasc Interv Radiol* 2001; **12**: 847–54.
25 Kawamura Y, Ikeda K, Hirakawa M *et al.* Efficacy of platinum analogue for advanced hepatocellular carcinoma unresponsive to transcatheter arterial chemoembolization with epirubicin. *Hepatol Res* 2009; **39**: 346–54.
26 Imai N, Ikeda K, Seko Y *et al.* Transcatheter arterial chemotherapy with miriplatin for patients with hepatocellular carcinoma and chronic renal failure. *Nihon Shokakibyo Gakkai Zasshi* 2011; **108**: 1872–8.
27 Kishimoto S, Miyazawa K, Terakawa Y *et al.* Cytotoxicity of cis-[((1R,2R)-1,2-cyclohexanediamine-N,N')bis (myristato)]-platinum (II) suspended in Lipiodol in a newly established cisplatin-resistant rat hepatoma cell line. *Jpn J Cancer Res* 2000; **91**: 1326–32.
28 Miyayama S, Yamashiro M, Shibata Y *et al.* Comparison of local control effects of superselective transcatheter arterial chemoembolization using epirubicin plus mitomycin C and miriplatin for hepatocellular carcinoma. *Jpn J Radiol* 2012; **30**: 263–70.
29 Shim JH, Kim KM, Lee YJ *et al.* Complete necrosis after transarterial chemoembolization could predict prolonged survival in patients with recurrent intrahepatic hepatocellular carcinoma after curative resection. *Ann Surg Oncol* 2010; **17**: 869–77.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clearance of hepatitis B surface antigen during long-term nucleot(s)ide analog treatment in chronic hepatitis B: results from a nine-year longitudinal study

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Abstract
Background Clearance of hepatitis B surface antigen (HBsAg) is considered the ultimate goal in chronic hepatitis B treatment. One treatment option is long-term nucleot(s)ide analog (NA) therapy. We followed a group of long-term NA therapy patients to evaluate the efficacy of this treatment in promoting clearance and longitudinal declines of HBsAg.
Method The study included 791 NA therapy patients who received lamivudine as their first drug. At the baseline, 442 patients were hepatitis B e antigen (HBeAg)+ and 349 were HBeAg−. All analyses were performed after separating the HBeAg+ and HBeAg− cohorts. Cox proportional hazards models were used to determine which factors were associated with HBsAg clearance.
Results HBsAg clearance was observed in 18 (4.1 %) of the HBeAg+ patients and 20 (5.7 %) of the HBeAg− patients at baseline, giving seroclearance rates of 6.4 and 6.9 %, respectively, over the nine-year study period. HBsAg clearance was influenced by several independent factors that varied according to HBeAg cohort. For HBeAg+ patients, these included previous interferon therapy, infection with hepatitis B virus (HBV) genotype A, a $\geq$0.5 log IU/mL decline in HBsAg level within six months, and clearance of HBeAg at six months. For HBeAg− patients, these included infection with HBV genotype A, decline in HBsAg at six months, and a baseline HBsAg level of <730 IU/mL.
Conclusion This study suggests that both direct antiviral potential and host immune response are needed to achieve HBsAg clearance by NA therapy. Viral genotype strongly influenced HBsAg clearance during NA therapy.

Keywords Hepatitis B surface antigen · Nucleot(s)ide analog · Lamivudine · Interferon

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Introduction

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, and one million people die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually [1, 2]. Recently, oral nucleot(s)ide analogs (NAs) have been used as a mainstay therapeutic strategy against chronic hepatitis B. Five such antiviral agents—lamivudine (LAM), entecavir (ETV), telbivudine, adefovir dipivoxil (ADV), and tenofovir disoproxil fumarate—which inhibit viral replication [e.g., hepatitis B virus DNA (HBV DNA) priming, reverse transcription of negative-stranded HBV DNA, and synthesis of positive-stranded HBV DNA] have been approved; these NAs vary in both the strength and the rapidity with which they suppress HBV DNA [3–10]. Sustained viral suppression by NA therapy can improve liver fibrosis and clinical outcomes of patients [11, 12]. LAM was the first NA to be approved to treat chronic hepatitis B in Japan, followed by ADV and ETV.

Responses to antiviral treatments can be evaluated by monitoring serum HBV DNA levels, hepatitis B e antigen (HBeAg) and antibody levels, and hepatitis B surface

antigen (HBsAg) and antibody levels. Serum HBsAg levels appear to reflect the amount of intrahepatic covalently closed circular DNA (cccDNA), which acts as a template for the transcription of viral genes [13–15]. Previous studies have shown that both interferon (IFN) and NA therapy result in a reduction of intrahepatic cccDNA [16, 17], suggesting that these treatments may be helpful in achieving the ultimate therapeutic goal of antiviral therapy for chronic hepatitis B (i.e., total clearance of HBsAg).

Very low rates of HBsAg clearance have been reported in the past [18–22]. Recent work has shown that over a one-year period, pegylated (PEG)-IFN therapy is more successful than ETV at reducing serum HBsAg [23]; furthermore, PEG-IFN therapy has also been reported to promote the complete clearance of HBsAg [24–27]. Several studies have detailed similar successes achieved by NA therapy but over relatively short (<5 years) treatment durations [18–20, 22, 28, 29]. The kinetics of HBsAg during long-term (>5 years) treatment remain unknown. NA therapy leads to time-dependent decreases in intrahepatic cccDNA and serum HBsAg levels if sustained viral suppression is longer term, and may therefore increase the rates of HBsAg clearance.

In order to evaluate this possibility empirically, we conducted a ten-year-long study in which we followed patients who received NA therapy initiated by the administration of LAM. We evaluated the resulting clearance and longitudinal declines of HBsAg using highly sensitive assays. Our aim was to determine whether long-term NA therapy can lead to HBsAg clearance, as suggested; if so, we also wished to elucidate the factors associated with its success.

Methods

Study population

Over a period of 12 years (September 1995 to September 2007), 949 consecutive patients who were chronically monoinfected with HBV (confirmed HBsAg positivity for at least six months), were treated with LAM monotherapy at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. The indication for antiviral therapy was abnormal ALT levels accompanying the increase in HBV DNA (over 4 log copies/mL) as a rule. However, in cases where ALT levels were normal, patients with advanced fibrosis were administered LAM. We did not treat patients without fibrosis who had low HBV DNA and normal ALT levels as a rule. We selected 791 patients for the final study after we had excluded all those who had been treated with LAM for <6 months, were co-infected with hepatitis C virus, had not provided sufficient serum samples, and/or had insufficient clinical records (Fig. 1). No patient was co-infected with human immunodeficiency virus in this cohort. Seven hundred ninety-one patients were enrolled in this cohort study. Of these 791 patients, 442 were HBeAg+ and 349 were HBeAg− at baseline. All analyses were performed after separating the HBeAg+ and HBeAg− cohorts. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institution's human research committee. This study has been registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN CTR) as the number UMIN000007993.

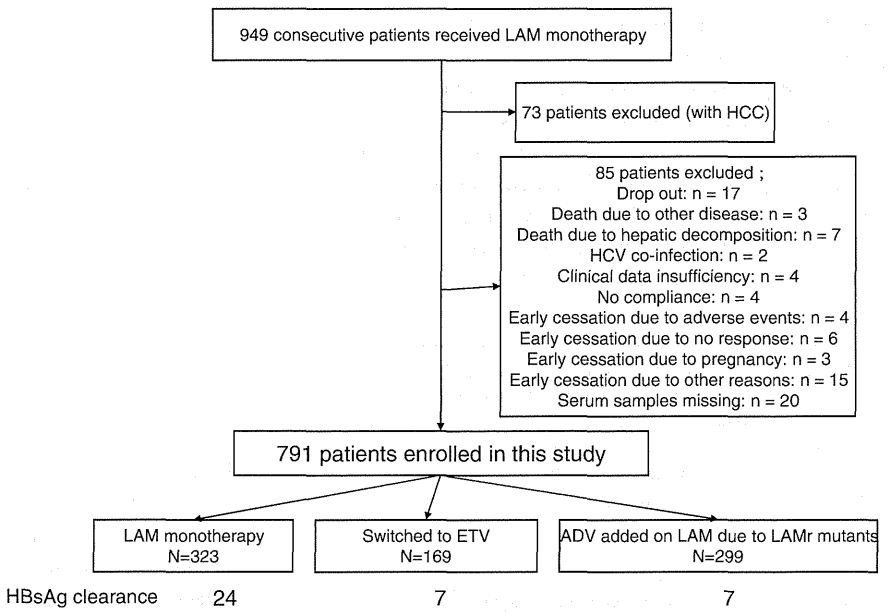

Fig. 1 Schematic of study protocol. *LAM* lamivudine, *HCC* hepatocellular carcinoma, *HCV* hepatitis C virus, *ETV* entecavir, *ADV* adefovir dipivoxil, *HBsAg* hepatitis B surface antigen

Antiviral therapy and drug resistance

All 791 patients received 100 mg LAM daily as an initial therapy, but a LAM-resistant rtM204I/V mutation developed in 439 (55 %) of these patients. Over time, 334 (42 %) individuals experienced an increase in HBV DNA (≥1 log copies/mL) [e.g., virological breakthrough (VBT)] and, as a result, 299 (98.5 %) individuals were also provided with ADV treatment (10 mg) added onto LAM as a rescue therapy. The remaining patients continued to receive LAM monotherapy and were lost to follow-up before the administration of ADV because of the lack of approval for ADV administration in Japan at the time. The resistant mutation for rtM204I/V was detected in 312 of 334 patients who experienced VBT using a commercial kit (as described below). Patients who had achieved an optimal or suboptimal virological response or who wished to participate in the clinical trial of ETV for LAM-refractory patients (ClinicalTrials.gov: NCT 1037166)—152 and 17 patients, respectively—switched from LAM to ETV (0.5 mg/day). Additionally, patients in whom subsequent ADV- or ETV-resistant mutants emerged received an optimal rescue therapy with other NAs (ETV + ADV combination for ADV resistance, and LAM + ADV combination for ETV resistance).

NA treatment was continued as a rule; median NA treatment duration was 75 months (25th–75th percentile, 55–102) in the HBeAg+ cohort and 92 months (67–119) in the HBeAg− cohort. Ultimately, 55 (7 %) of the 791 patients discontinued treatment; 16 of these individuals terminated treatment after achieving HBsAg seroclearance. Follow-ups were conducted for all patients, regardless of length of treatment, for as long as possible.

Clinical data collection and follow-ups

Data on patient characteristics, biochemistry, hematology, virology, histology, and previous treatments were collected and registered in our institute's database at the time of patient enrollment. Prior to beginning LAM, all patients were surveyed about the presence of a family history of HBV infection. Data on treatment dose and duration of previous IFN therapy were collected from our hospital's IFN therapy database or requested from other hospitals as necessary. Complete details on the previous treatment were lacking for 29 (9.7 %) of 297 patients who received IFN therapy before starting LAM.

At least every 1–3 months, liver function and virological markers of HBV infection were measured in all patients. All serum HBsAg titers were measured from frozen serum samples collected at six months, one year, three years, five years, and once annually for 6–10 years, and then stored at −80 °C. The day of HBsAg clearance was defined by the measurement in consecutive available serum samples before it was undetected in subsequent samples. A genotypic analysis of drug resistance was performed in cases of insufficient virological response or VBT, defined as an increase in serum HBV DNA levels ≥1 log above the nadir measured after the initial virological response. Cirrhosis was diagnosed by laparoscopy, liver biopsy, or clinical data such as imaging modalities and portal hypertension. The primary outcome for this study was HBsAg clearance. The endpoint of the follow-up was HBsAg clearance or last visit before January 2011.

Markers of HBV infection

Serum HBsAg titers were measured using ARCHITECT HBsAg QT assay kits (Abbott Laboratories, Tokyo, Japan), which have a lower limit of detection of 0.05 IU/mL and an upper limit of detection of 250 IU/mL. To expand the upper range from 250 to 125,000 IU/mL, serum samples, going off the scale, were diluted stepwise to 1:20 and 1:500 with ARCHITECT diluents as the product document described. HBeAg was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantified using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6–7.6 log copies/mL, or COBAS TaqMan HBV v.2.0 (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.1–9.0 log copies/mL. A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to serologically determine HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific to each of the seven major genotypes (A–G). YMDD mutants (rt M204I/V) were determined by polymerase chain reaction-based enzyme-linked mini-sequence assay with a commercial kit (Genome Science Laboratories, Tokyo, Japan).

Statistical analyses

Categorical data were compared between groups using chi-square or Fisher's exact tests. Continuous variables with a nonparametric distribution were analyzed with Mann–Whitney *U* tests, while those with a parametric distribution were analyzed with Student's *t* tests. When appropriate, Kruskal–Wallis tests were used to conduct pairwise comparisons of specific variables. Cox regression analyses were used to assess which variables were significantly associated with HBsAg clearance. Cut-off values were provided using the area under the receiver operating characteristic curve (ROC) only after rejecting the null hypothesis for the ROC curve. All baseline factors that were found to be significantly associated with HBsAg clearance by univariate analysis

were entered into a multivariate analysis. Independent baseline factors associated with clearance of HBsAg were calculated using a stepwise Cox regression analysis. We then performed a time-dependent Cox regression to analyze independent factors associated with HBsAg while adjusting for on-treatment factors and independent baseline factors. Three covariates of the on-treatment response factors—emergence of rtM204I/V mutants, VBT, and biochemical breakthrough—were set as the time-dependent covariates. Cumulative HBsAg clearance rates were analyzed using the Kaplan–Meier method; differences in the resulting curves were tested using log-rank tests. We performed Cox regression analysis, Kaplan–Meier curve analysis, and HBsAg kinetics analysis for no more than nine years, as the number of patients with a long-term follow-up of over ten years was too small to permit analysis [30]. Bonferroni adjustments were used to correct for the number of different ways a single predictor variable can be split. Significance was defined as $P < 0.05$ for all two-tailed tests. Data analysis was performed with IBM SPSS version 19.0 software (IBM Corp., Armonk, NY, USA).

Results

Patient characteristics

Thirty-eight (4.8 %) of 791 patients successfully cleared HBsAg. Of these, 24 had received LAM, 7 had switched to ETV treatment, and 7 had been treated with both LAM and ADV (Fig. 1). Of the 38 patients who achieved HBsAg clearance, 18 were HBeAg+, whereas 20 were HBeAg− at baseline. Table 1 provides a comparison of the baseline and on-treatment characteristics between patients who were and were not able to successfully clear HBsAg (all patients, HBeAg+ and − cohorts, respectively). In the HBeAg+ cohort, baseline characteristics that were significantly associated with HBsAg clearance included previous IFN therapy, HBV genotype, HBV DNA, and AST and ALT levels; in the HBeAg− cohort, significant characteristics included HBV genotype and HBsAg levels. Significant on-treatment characteristics in the HBeAg+ cohort included decline in HBsAg, clearance of HBeAg, and decline in HBV DNA to <2.6 log copies/mL at six months;

Table 1 Baseline, demographic, and on-treatment characteristics of patients with and without HBsAg seroclearance

Characteristics	All patients ($n = 791$)	HBeAg+ at baseline ($n = 442$)			HBeAg− at baseline ($n = 349$)		
		Persistently HBsAg+ ($n = 424$)	HBsAg seroclearance ($n = 18$)	P	Persistently HBsAg+ ($n = 329$)	HBsAg seroclearance ($n = 20$)	P
Baseline							
Age[a] (years) (SD)	43 (11.1)	41 (11.2)	44 (10.5)	0.177	47 (10.3)	46 (10.3)	0.899
Gender (male:female)	627:164	329:95	16:2	0.385	265:64	16:4	1.000
Race				0.446			
Japanese	768 (97)	411 (97)	17 (94)		320 (97)	20 (100)	1.000
Non-Japanese (%) (Asian:Caucasian)	23 (3) (21:2)	13 (3) (20:2)	1 (3) (1:0)		9 (3) (20:2)	0 (3) (1:0)	
Family history of HBV infection	539 (68)	311 (73)	10 (56)	0.107	208 (63)	10 (50)	0.238
Previous IFN therapy	297 (38)	167 (39)	15 (83)	**<0.001**	106 (32)	9 (45)	0.326
IFN duration (weeks)	27 (20–58)	26 (18–53)	52 (21–79)	0.214	32 (22–89)	23 (14–72)	0.457
Duration from the end of IFN to start of lamivudine (weeks)	50 (3–189)	26 (7–124)	37 (2–89)	0.505	119 (3–316)	102 (18–289)	0.746
Previous NA therapy	34 (4)	21 (5)	2 (11)	0.239	10 (3)	1 (5)	0.483
Presence of cirrhosis	169 (21)	76 (18)	2 (11)	0.752	87 (26)	4 (20)	0.610
HBV genotype				**<0.001**			**<0.001**
A	28 (3.5)	14 (3.3)	6 (33)		6 (1.8)	2 (10)	
B	67 (8.5)	16 (3.8)	0 (0)		48 (14.6)	3 (15)	
C	664 (83.9)	374 (88.2)	12 (67)		265 (80.5)	13 (65)	
D	3 (0.4)	2 (0.4)	0 (0)		0 (0)	1 (5)	
F	2 (0.3)	2 (0.4)	0 (0)		0 (0)	0 (0)	
Unclassified/missing	27 (3.4)	16 (3.8)	0 (0)		10 (3.0)	1 (5)	

Table 1 continued

Characteristics	All patients (n = 791)	HBeAg+ at baseline (n = 442)			HBeAg− at baseline (n = 349)		
		Persistently HBsAg+ (n = 424)	HBsAg seroclearance (n = 18)	P	Persistently HBsAg+ (n = 329)	HBsAg seroclearance (n = 20)	P
Baseline HBV DNA (log copies/mL)	7.0 (5.8–8.0)	7.6 (6.7–8.2)	8.0 (7.5–8.4)	**0.027**	6.3 (5.2–7.2)	6.1 (5.0–7.0)	0.652
Baseline HBsAg level (IU/mL)	2530 (907–6590)	3910 (1690–12300)	5280 (943–67600)	0.331	1590 (599–3050)	529 (58–1610)	**0.004**
Baseline AST level (IU/L)	74 (48–135)	81 (52–165)	201 (78–666)	**0.011**	66 (42–113)	57 (39–96)	0.694
Baseline AST level (×ULN)	2.2 (1.5–4.1)	2.5 (1.6–5.0)	6.1 (2.3–20.2)	**0.011**	2.0 (1.3–3.4)	1.7 (1.2–2.9)	0.736
Baseline ALT level (IU/L)	115 (63–252)	130 (72–290)	326 (104–775)	**0.021**	101 (56–194)	101 (55–215)	0.904
Baseline ALT level (×ULN)	3.0 (1.7–6.4)	3.5 (1.9–7.8)	7.8 (2.5–20.3)	**0.040**	2.6 (1.4–5.2)	2.6 (1.4–5.2)	0.955
Baseline total bilirubin level (mg/dL)	0.8 (0.6–1.1)	0.8 (0.5–1.1)	0.9 (0.6–1.9)	0.117	0.7 (0.6–1.0)	0.8 (0.6–0.9)	0.556
Platelet count[a] (10^5/mm^3) (SD)	16.1 (5.7)	16.5 (6.1)	14.7 (3.5)	0.221	15.6 (5.1)	17.7 (6.9)	0.216
On-treatment response							
Decline of HBsAg level (≥0.5 log IU/mL within six months)	97 (1)	67 (16)	13 (72)	**<0.001**	11 (3)	6 (30)	**<0.001**
HBeAg positive → clearance within six months	109 (14)	94 (22)	10 (56)	**0.005**	NA	NA	
Undetectable HBV DNA (<400 copies/mL) at six months	532 (67)	221 (52)	15 (83)	**0.014**	277 (84)	19 (95)	0.330
Emergence of rtM204I/V mutants	439 (55)	251 (59)	9 (50)	0.469	170 (52)	9 (45)	0.646
Viral breakthrough due to mutants	334 (42)	216 (51)	5 (28)	0.055	108 (33)	5 (25)	0.473
Biochemical breakthrough due to mutants	318 (40)	200 (47)	5 (28)	0.146	108 (33)	5 (25)	0.473

Except where marked with a superscript letter a, values are expressed as the median and 25th–75th percentiles (parenthetically), or number and percentage (parenthetically). ULN; AST = 33 IU/L, ALT = 42 IU/L (male), and 27 IU/L (female). *Asterisks* indicate data displayed as mean values and standard deviations. *Bold text* indicates statistically significant P values

the only significant characteristic in the HBeAg− cohort was a decline in HBsAg within six months. ROC curve analysis confirmed a cut-off value of 0.5 log IU/mL for a decline in HBsAg level within six months in the HBeAg+ and − cohorts [area under the curve = 0.810 (95 % CI 0.673–0.947) (HBeAg+ cohort) and 0.760 (95 % CI 0.611–0.909) (HBeAg− cohort)].

LAM-resistant rtM204I/V mutants were detected in 439 (55.5 %) of 791 patients. Of these, 334 (42.2 % of all patients) also developed VBT accompanied by an increase in HBV DNA (≥1 log copies/mL). The rate of VBT was marginally significantly lower in the HBsAg clearance group in the HBeAg+ cohort (Table 1).

Factors associated with HBsAg clearance

The overall cumulative rates of HBsAg clearance were 0.2 % at one year, 1.2 % at three years, 2.6 % at five years, 4.2 % at seven years, and 6.4 % at nine years in the HBeAg+ cohort; and 0.6 % at one year, 0.9 % at three - years, 2.2 % at five years, 5.2 % at seven years, and 6.9 % at nine years in the HBeAg− cohort. Univariate Cox

Table 2 Baseline and on-treatment response factors associated with HBsAg clearance, as determined by time-dependent univariate and multivariate analyses at year 9 (HBeAg+ cohort)

Variable	Univariate		Multivariate	
	HBsAg clearance rate ratio (95 % CI)	*P*	HBsAg clearance rate ratio (95 % CI)	*P*
Baseline factors				
Age (≥50 years)	1.36 (0.48–3.86)	0.564		
Gender (F)	0.51 (0.12–2.23)	0.371		
Family history of HBV infection	0.42 (0.16–1.09)	0.074		
Previous IFN therapy	**5.60 (1.61–19.5)**	**0.007**	**6.15 (1.69–22.4)**	**0.006**
Previous NA therapy	2.42 (0.55–10.6)	0.242		
Presence of cirrhosis	0.85 (0.52–1.40)	0.527		
HBV genotype (A)	**3.64 (2.21–5.99)**	**<0.001**	**3.18 (1.80–5.62)**	**<0.001**
HBV DNA (≥6.0 log copies/mL)	2.56 (0.34–19.3)	0.362		
HBsAg (<730 IU/mL)	1.57 (0.51–4.81)	0.432		
AST (≥4.5 × ULN)	**4.53 (1.68–12.2)**	**0.003**		
ALT (≥7.2 × ULN)	**3.56 (1.35–9.36)**	**0.010**		
Total bilirubin (≥1.5 mg/dL)	2.63 (0.92–7.46)	0.070		
Platelet count (<1.2 × 10^5/mm^3)	0.58 (0.13–2.59)	0.476		
On-treatment response factors				
Decline of HBsAg level (≥0.5 log IU/mL within six months)	**15.8 (5.14–48.5)**	**<0.001**	**18.6 (5.78–60.0)**	**<0.001**
HBeAg positive → clearance within six months	**4.33 (1.65–11.4)**	**0.003**	**2.95 (1.04–8.39)**	**0.042**
Undetectable HBV DNA (<400 copies/mL) at six months	**3.95 (1.14–13.7)**	**0.031**		
Emergence of rtM204I/V mutants[a]	0.88 (0.32–2.44)	0.802		
Viral breakthrough due to mutants[a]	**0.32 (0.10–1.00)**	**0.050**		
Breakthrough hepatitis due to mutants[a]	0.41 (0.13–1.31)	0.134		

[a] Time-dependent covariates. *Bold text* indicates statically significant *P* valuesVariables analyzed in multivariate analysis: previous IFN therapy, HBV genotype, ALT, decline of HBsAg levels, HBeAg clearance within six months, undetectable HBV DNA at six months, and viral breakthrough due to mutants (time-dependent covariate)

regression analysis identified four baseline characteristics and four on-treatment responses that were associated with HBsAg clearance in the HBeAg+ cohort (Table 2), and two baseline characteristics and two on-treatment responses in the HBeAg− cohort (Table 3). ROC curve analysis provided the optimal cut-off values and indices for the prediction of HBsAg clearance. ROC curve analysis confirmed cut-off indices of 4.5 × ULN for AST and 7.2 × ULN for ALT for HBsAg clearance in the HBeAg+ cohort [area under the curve = 0.677 (95 % CI 0.524–0.830) (AST) and 0.643 (95 % CI 0.503–0.783) (ALT)]. Meanwhile, ROC curve analysis confirmed a cut-off value of 730 IU/mL (2.86 log IU/mL) for HBsAg for HBsAg clearance in the HBeAg− cohort [area under the curve = 0.696 (95 % CI 0.556–0.836)]. Time-dependent multivariate Cox regression analysis identified two significant baseline characteristics and two on-treatment responses related to HBsAg clearance: previous IFN therapy, infection with HBV genotype A, a decline in HBsAg level of ≥0.5 log IU/mL within six months, and HBeAg clearance within six months in the HBeAg+ cohort (Table 2). In the HBeAg− cohort, two baseline characteristics and one on-treatment response were identified in multivariate analysis: infection with HBV genotype A, HBsAg level of <730 IU/mL (2.86 log IU/mL), and a decline in HBsAg level of ≥0.5 log IU/mL within six months (Table 3).

Association between HBV genotype and HBsAg clearance

We performed a detailed analysis of the association between HBV genotype and HBsAg clearance in patients treated with NAs. Median baseline HBsAg levels were 4.7 log IU/mL (25th–75th percentile, 4.4–5.1) among patients with genotype A, 3.8 (3.5–4.2) among patients with genotype B, and 3.5 (3.2–4.0) among patients with genotype C in the HBeAg+ cohort (Fig. 2a); and 3.7 (2.5–4.1) in patients with genotype A, 2.9 (2.6–3.5) in patients with genotype B, and 3.2 (2.8–3.5) in patients with genotype C in the HBeAg− cohort (Fig. 2b). HBeAg+ patients with genotype A had higher baseline HBsAg levels than those with genotypes B or C ($P < 0.001$) (Fig. 2a). There were no significant differences in baseline HBsAg levels between the genotypes in the HBeAg− cohort.

Table 3 Baseline and on-treatment response factors associated with HBsAg clearance, as determined by time-dependent univariate and multivariate analyses at year 9 (HBeAg− cohort)

Variable	Univariate		Multivariate	
	HBsAg clearance rate ratio (95 % CI)	*P*	HBsAg clearance rate ratio (95 % CI)	*P*
Baseline factors				
Age (≥50 years)	1.39 (0.54–3.60)	0.498		
Gender (F)	0.98 (0.28–3.40)	0.971		
Family history of HBV infection	0.49 (0.19–1.27)	0.140		
Previous IFN therapy	0.88 (0.32–2.38)	0.797		
Previous NA therapy	2.41 (0.32–18.2)	0.394		
Presence of cirrhosis	0.71 (0.43–1.16)	0.173		
HBV genotype (A)	**2.79 (1.33–5.85)**	**0.007**	**2.73 (1.29–5.81)**	**0.009**
HBV DNA (≥6.0 log copies/mL)	1.16 (0.43–3.14)	0.772		
HBsAg (<730 IU/mL)	**3.91 (1.59–9.52)**	**0.003**	**4.90 (1.85–10.6)**	**0.001**
AST (≥4.5 × ULN)	1.76 (0.57–5.40)	0.324		
ALT (≥7.2 × ULN)	1.89 (0.62–5.81)	0.265		
Total bilirubin (≥1.5 mg/dL)	1.18 (0.27–5.20)	0.825		
Platelet count (<1.2 × 10^5/mm^3)	0.77 (0.17–3.55)	0.733		
On-treatment response factors				
Decline of HBsAg level (≥0.5 log IU/mL within six months)	**11.5 (4.24–31.0)**	**<0.001**	**16.9 (5.89–48.4)**	**<0.001**
Undetectable HBV DNA (<400 copies/mL) at six months	2.78 (0.37–20.8)	0.322		
Emergence of rtM204I/V mutants[a]	0.64 (0.23–1.79)	0.392		
Viral breakthrough due to mutants[a]	0.72 (0.23–2.29)	0.581		
Breakthrough hepatitis due to mutants[a]	0.65 (0.21–2.06)	0.465		

[a] Time-dependent covariates. *Bold text* indicates statically significant *P* values

Variables analyzed in multivariate analysis: HBV genotype, baseline HBsAg, decline of HBsAg levels

HBsAg kinetics over time in the HBeAg+ and − cohorts are shown in Fig. 2c, d, respectively. Among patients with genotype A in the HBeAg+ cohort, the median HBsAg change from baseline was −0.44 log IU/mL at six months, −0.56 at one year, −0.58 at three years, −1.08 at five years, and −1.33 at six years. Among patients with genotype B in the HBeAg+ cohort, median changes were −0.30 log IU/mL at six months, −0.30 at one year, −0.43 at three years, and −0.46 at five years. Kinetics were not calculated for some groups (genotype A at seven years, genotype B at six years) because the number of patients was too small. Finally, among patients with genotype C in the HBeAg+ cohort, median changes were 0.00 log IU/mL at six months, 0.03 at one year, −0.08 at three years, −0.29 at five years, −0.53 at six - years, −0.62 at seven years, −0.70 at eight years, and −0.82 at nine years. Genotype had a significant effect on the slopes between data collection points at six months and six years. In the HBeAg+ cohort, declines were faster in patients with genotype A than in those with genotypes B or C. HBeAg− patients with genotype A displayed a median HBsAg change from baseline of 0.05 log IU/mL at six months, 0.05 at one year, −0.11 at three years, −0.21 at five years, and −0.26 at six years. Among patients with genotype B in the HBeAg− cohort, median changes were −0.03 log IU/mL at six months, −0.06 at one year, −0.25 at three years, −0.31 at five years, −0.51 at six years, −0.62 at seven years, −0.66 at eight years, and −0.61 at nine years. Among patients with genotype C in the HBeAg− cohort, median changes were 0.03 log IU/mL at six months, 0.04 at one year, −0.08 at three years, −0.19 at five years, −0.32 at six years, −0.39 at seven years, −0.46 at eight years, and −0.62 at nine years. The decline was slightly faster in patients with genotype B than in those with genotypes A and C in the HBeAg− cohort.

We investigated whether HBsAg clearance were influenced by genotype or baseline HBeAg. Cumulative HBsAg clearance rates in the HBeAg+ cohort were as follows: 15 % at year 3, and 35 % at year 5 in patients with genotype A; 0 % over all years in patients with genotype B; and 0.6 % at year 3, 1.2 % at year 5, and 5.4 % at year 9 in patients with genotype C (Fig. 2e). In the HBeAg− cohort, clearance rates were 12 % at year 3, and 25 % at year 5 in patients with genotype A; 0 % at year 3, 0 % at year 5, and 11.5 % at year 9 in patients with genotype B; and 0.4 % at year 3, 1.6 % at year 5, and 5.1 % at year 9 in

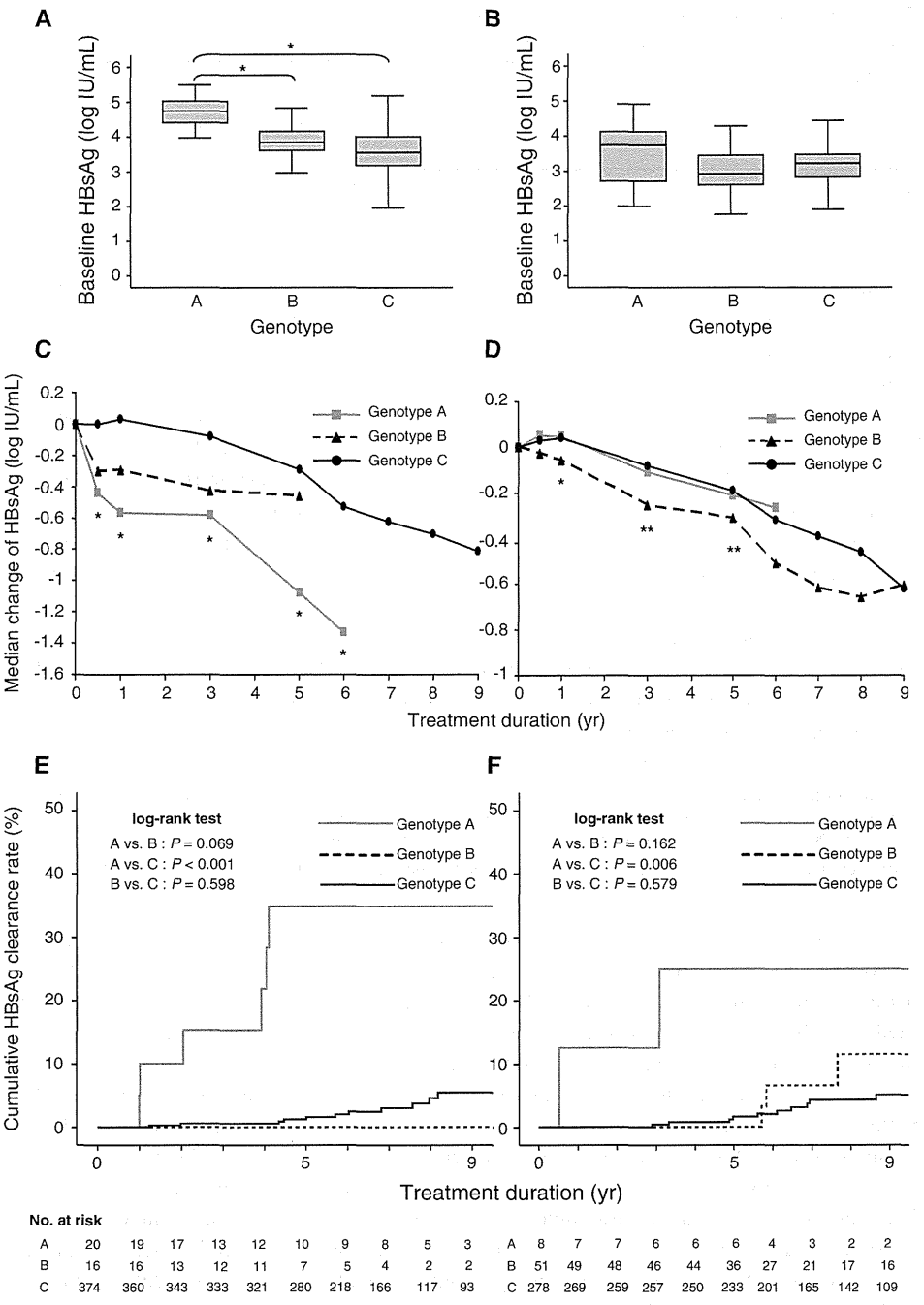

Fig. 2 **a** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg+ cohort). The *asterisk* (*) indicates a statistical significance of $P < 0.001$, as determined by the Mann–Whitney U test and Bonferroni correction. **b** Box plot of baseline HBsAg levels in patients with different HBV genotypes (HBeAg− cohort). **c** Median change in HBsAg level from baseline in patients with different HBV genotypes (HBeAg+ cohort). A *single asterisk* (*) indicates $P < 0.001$, as determined by the Kruskal–Wallis test. **d** Median change in HBsAg level from baseline in patients with different HBV genotypes (HBeAg− cohort). A *single asterisk* (*) indicates $P < 0.001$ and a *double asterisk* (**) indicates $P < 0.02$, as determined by the Kruskal–Wallis test. **e** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with different HBV genotypes (HBeAg+ cohort). Cumulative HBsAg clearance rates were significantly higher among patients with genotype A (log-rank test; A vs. B: $P = 0.069$, A vs. C: $P < 0.001$, B vs. C: $P = 0.598$, after Bonferroni correction). **f** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with different HBV genotypes (HBeAg− cohort). Cumulative HBsAg clearance rates were significantly higher among patients with genotype A (log-rank test; A vs. B: $P = 0.169$, A vs. C: $P = 0.006$, B vs. C: $P = 0.579$, after Bonferroni correction)

patients with genotype C (Fig. 2f). Clearance rates were significantly higher in patients with genotype A than in those with genotype C ($P < 0.001$ in the HBeAg+ cohort, $P = 0.006$ in the HBeAg− cohort).

Association between on-treatment response and subsequent HBsAg clearance

We stratified patients into three groups according to the amount of HBsAg decline within the first six months of treatment; this allowed us to evaluate the impact of on-treatment response factors on the clearance of HBsAg. The stratifications were as follows: rapid decline (≥1.0 log IU/mL), intermediate decline (0.5–1.0 log IU/mL), and slow decline or steady (<0.5 log IU/mL). Cumulative HBsAg clearance rates in the HBeAg+ cohort were 11 % at year 3, and 40 % at year 5 in the rapid decline group; 0 % at year 3, 2.2 % at year 5, and 13 % at year 9 in the intermediate decline group; and 0 % at year 3, 0 % at year 5, and 2.9 % at year 9 in the slow decline or steady group (Fig. 3a).

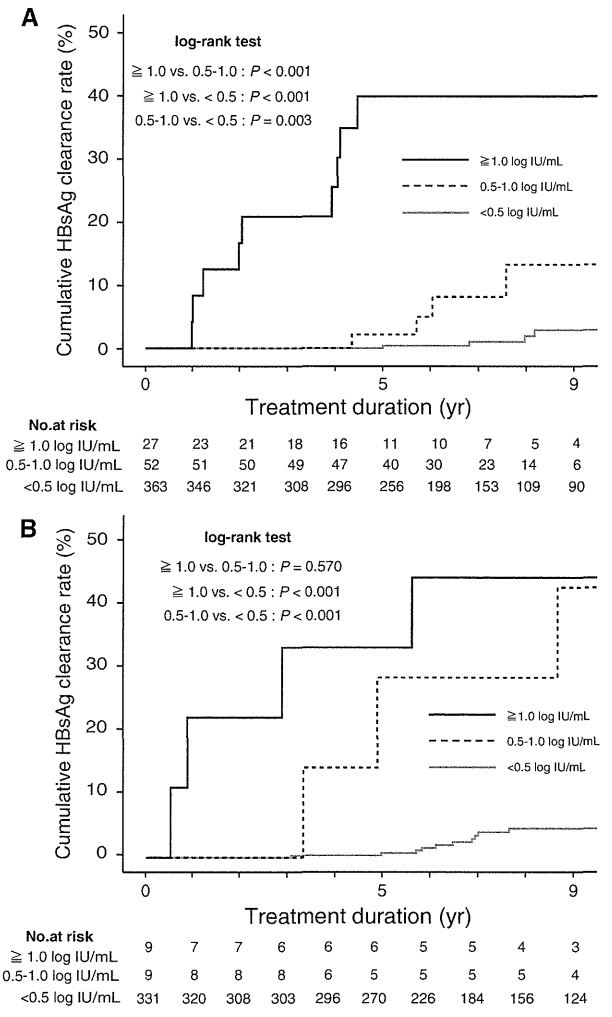

Fig. 3 **a** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg+ cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank test; rapid vs. intermediate: $P < 0.001$, rapid vs. slow: $P < 0.001$, intermediate vs. slow: $P = 0.003$, after Bonferroni correction). **b** Kaplan–Meier life table showing cumulative HBsAg clearance rates in patients with varying rates of HBsAg decline within the first six months (HBeAg− cohort). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group (log-rank test; rapid vs. intermediate: $P = 0.570$, rapid vs. slow: $P < 0.001$, intermediate vs. slow: $P < 0.001$, after Bonferroni correction)

Cumulative HBsAg clearance rates in the HBeAg− cohort were 33 % at year 5, and 44 % at year 7 in the rapid decline group; 0 % at year 3, 29 % at year 5, and 43 % at year 9 in the intermediate decline group; and 0.3 % at year 3, 0.7 % at year 5, and 4.6 % at year 9 in the slow decline or steady group (Fig. 3b). Clearance rates were highest in the rapid decline group, followed by the intermediate decline group and the slow or steady group in both the HBeAg+ and HBeAg− cohorts. The decline of HBsAg within the first six months was a strong predictor of HBsAg clearance.

Viral breakthrough and subsequent HBsAg clearance

Although VBT was not associated with HBsAg clearance in the multivariate model, as described above, HBsAg clearance was observed in ten patients who experienced VBT (five patients in the HBeAg+ cohort and five in the HBeAg− cohort). All ten patients achieved clearance of HBsAg after VBT occurred. Six of these patients received ADV added on to LAM for VBT, and subsequently achieved clearance of HBsAg (five patients in the HBeAg+ cohort and one in the HBeAg− cohort). The other four patients spontaneously recovered from VBT while continuing to receive LAM monotherapy, and subsequently achieved clearance of HBsAg (one patient in the HBeAg+ cohort and three in the HBeAg− cohort). LAM-resistant mutant strains (M204I/V mutants) were detected in nine patients in whom VBT occurred. HBV DNA negativity continued for the follow-up period after HBsAg clearance in these ten patients. The typical clinical and virological courses of two representative patients who achieved HBsAg clearance after VBT are shown in Fig. 4a, b.

Virological courses after discontinuation of NAs

Sixteen (42.1 %) of 38 patients with HBsAg clearance discontinued NA treatment due to HBsAg clearance. Median interval between HBsAg clearance and discontinuation of NAs was nine months (range 2–29 months). Median follow-up period after discontinuation of NAs was 24 months (range 7–171) in these patients. No relapses of serum HBsAg or HBV DNA were observed during the follow-up period. Serum anti-HBs appeared in 12 (75 %) of the 16 patients who discontinued NAs. Median time to the appearance of anti-HBs after HBsAg clearance was 16 months (range 2–92) in patients who discontinued NAs. Two of 22 patients who continued NAs with HBsAg clearance had the appearance of anti-HBs, and median time to the appearance of anti-HBs after HBsAg clearance was two and seven months in these two patients, respectively.

Discussion

We found that three baseline factors and two on-treatment response factors are associated with HBsAg clearance in patients who begin treatment with LAM and continue with long-term NA therapy. HBV genotype and the decline in HBsAg over the first six months were associated with