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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.]

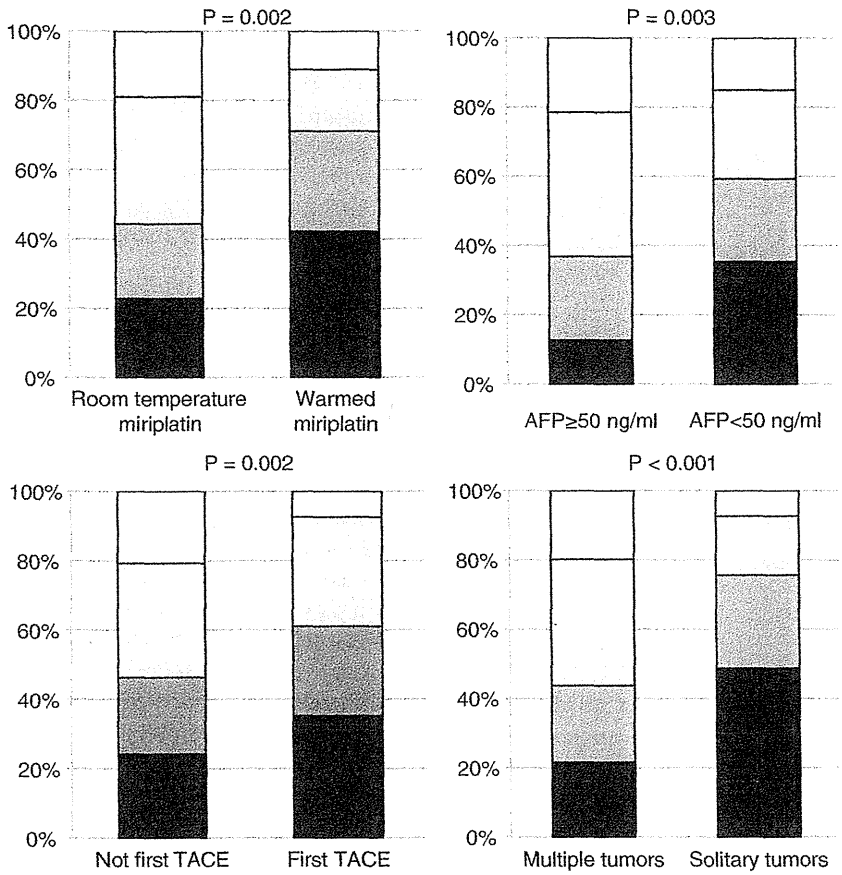

Figure 1 Efficacy of transcatheter arterial chemoembolization (TACE) using miriplatin in patients with hepatocellular carcinoma according to miriplatin temperature, serum α-fetoprotein (AFP) level, history of TACE and tumor number. Complete response (CR) and partial response (PR) rates were significantly 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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ORIGINAL ARTICLE—LIVER, PANCREAS, and BILIARY TRACT

Long-term efficacy and emergence of multidrug resistance in patients with lamivudine-refractory chronic hepatitis B treated by combination therapy with adefovir plus lamivudine

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Abstract
Background Few studies have investigated the emergence of multidrug resistance to adefovir dipivoxil (ADV) plus lamivudine (LAM) combination therapy for patients with LAM-refractory chronic hepatitis B (CHB). In this retrospective study, we investigated the long-term clinical course of these patients with or without multidrug resistance mutations.
Methods We analyzed 406 Japanese patients with LAM-refractory CHB treated with combination therapy with follow-up for a median of 5.4 (0.5–9.5) years. Multidrug resistance of hepatitis B virus (HBV) DNA was analyzed using direct sequencing or cloning methods at baseline and viral breakthrough or insufficient decline during combination therapy.
Results Ratio of patients with undetectable serum HBV DNA levels (<2.6 log copies/mL) during combination therapy was 63, 72, 75, 79, 82, 80 and 85 % at years 1 through 7, respectively. Substitutions associated with multidrug resistance were identified in 11 patients (2.7 %) at baseline, and in 12 patients (3 %) during therapy. HBV DNA levels of patients with rtA181S mutation at baseline and emergence of rtA181T + rtN236T double mutation or a wide variety of mutations during combination therapy could not be suppressed. Moreover, using ultra-deep sequencing, rtA181T/V mutations were detected at baseline in 7 of 10 patients with emergent multidrug resistance during combination therapy, although 6 of these 7 patients had very low frequency (<1 %) variants.
Conclusion Long-term ADV plus LAM combination therapy is effective in LAM-refractory patients. However, HBV DNA levels of the patients with multidrug resistance at baseline or during combination therapy sometimes could not achieve complete suppression or were re-elevated after a decrease.

Keywords Adefovir dipivoxil · Lamivudine · Hepatitis B virus · Ultra-deep sequence · Multidrug resistance

Abbreviations

HBV	Hepatitis B virus
IFN	Interferon
NA	Nucleoside/nucleotide analogues
LAM	Lamivudine
ADV	Adefovir dipivoxil
ETV	Entecavir
TDF	Tenofovir disoproxil fumarate
CHB	Chronic hepatitis B
HBeAg	Hepatitis B e antigen
ALT	Alanine aminotransferase
HBsAg	Hepatitis B surface antigen
PCR	Polymerase chain reaction
CLEIA	Chemiluminescent enzyme immunoassay
rt	Reverse transcriptase

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VBT Viral breakthrough
AST Aspartate aminotransferase
CI Confidence interval
Pt Patient

Introduction

Hepatitis B virus (HBV) infection is a common disease that can induce a chronic carrier state, and is associated with the risk of developing progressive disease and hepatocellular carcinoma [1]. Interferon (IFN) and several nucleoside/nucleotide analogues (NA) such as lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), and tenofovir disoproxil fumarate (TDF) are currently approved for treatment of chronic hepatitis B (CHB) in most countries [2–8]. Successful treatment of CHB with clearance of hepatitis B e antigen (HBeAg), reduction in serum HBV DNA levels, and normalization of alanine aminotransferase (ALT) levels are associated with favorable long-term outcomes, independent of the antiviral drug used [9–11].

LAM is effective in suppressing HBV replication, improving transaminase levels and liver histology, and enhancing the rate of loss of HBeAg. A major problem with the long-term use of lamivudine, however, is its potential to induce viral resistance, with associated increases in HBV DNA and serum transaminases [3, 12, 13]. ADV is reportedly effective in suppressing HBV replication and is approved as a standard therapy in LAM-resistant patients in Japan [14, 15]. However, data concerning the long-term efficacy of ADV treatment in LAM-resistant CHB patients remain limited.

Although both experimental and clinical studies have shown that ADV suppresses not only wild-type but also LAM-resistant strains, the potential for ADV-resistance mutation has emerged. Selection of the rtA181V/T or rtN236T mutant was associated with ADV [13, 16]. Moreover, we previously reported that the emergence of ADV-resistant mutations before and during combination therapy for a period of 2 years was rare [17]. However, ADV-resistant mutations emerging before and during combination therapy might be caused by a poor response to therapy. Moreover, long-term clinical and virological data concerning ADV- or ETV-resistant mutations in LAM-resistant CHB patients receiving long-term ADV plus LAM combination therapy are limited.

The aims of this study were to evaluate the long-term efficiency of ADV plus LAM combination therapy based on virological response (VR), HBeAg clearance, and Hepatitis B surface antigen (HBsAg) clearance, and to investigate the emergence of ADV-, ETV-, or TDF-resistant (or multidrug resistant) mutations before and during combination therapy, and the clinical course of these patients.

Patients and methods

Patients

A total of 406 consecutive adult Japanese patients with chronic HBV infection were treated with ADV in addition to ongoing LAM treatment from 2002 at Toranomon Hospital (Table 1). Several of these patients were included in previous reports [14, 15, 17, 18]. Enrollment in this study and the start of ADV treatment were determined by the following criteria. First, an increase in serum HBV DNA levels of $\geq$1 log copies/mL during LAM treatment compared with the nadir of initial antiviral efficacy on at least two consecutive occasions, or a serum HBV DNA level of $\geq$5 log copies/mL after 1 year of LAM monotherapy; and second, no history of treatment with other NAs such as ETV or TDF. Exclusion criteria were a serum creatinine level $\geq$1.2 mg/dL; coinfection with hepatitis C virus or HIV; and history of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, or metabolic liver disease. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical

Table 1 Characteristics of patients at the commencement of adefovir dipivoxil plus lamivudine combination therapy

Demographic data	
Total number	406
Sex (female/male)	86/320
Age, years (range)	48 (25–78)
Duration of treatment, years (range)	5.4 (0.5–9.5)
History of IFN therapy (+/−)	157/249
Laboratory data	
Aspartate aminotransferase, IU/L (range)	54 (12–1413)
Alanine aminotransferase, IU/L (range)	76 (9–1563)
Bilirubin, mg/dL (range)	0.7 (0.2–15.5)
Albumin, g/dL (range)	3.9 (1.9–4.7)
Platelets, $\times 10^3/\mu$L (range)	160 (28–452)
Staging of liver histology (CH/LC)	325/81
Serum HBV DNA, log copies/mL (range)	6.7 (<2.6 to >7.6)
HBeAg, positive/negative/unknown	208/193/5
HBV genotype (A/B/C/D/F)	14/25/364/2/1
rtM204 mutant (%)	365 (90 %)

Values are expressed as the median and range in parentheses, or number and percentage in parentheses

IFN interferon, *HBV* hepatitis B virus, *CH* chronic hepatitis, *LC* liver cirrhosis, *HBeAg* hepatitis B e antigen

Committee (approval no. 714). Informed consent was obtained from all patients.

Patients received a single daily oral administration of ADV 10 mg, in addition to ongoing LAM treatment (100 mg/day). The dosing interval of ADV was modified by the attending physician when serum creatinine level increased to >1.2 mg/dl. Liver cirrhosis was defined by the presence of stage 4 fibrosis on histopathological examination and/or clinical evidence of portal hypertension.

Blood tests and serum viral markers

Routine biochemical tests were performed using standard procedures before and during therapy at least once every 3 months. Levels of HBsAg, HBeAg, and anti-HBe were determined using radioimmunoassay kits (Abbot Diagnostics, Chicago, IL, USA) or Chemiluminescent enzyme immunoassay (CLEIA; Lumipulse System, Fujirebio, Inc. Tokyo, Japan). Serum HBV DNA was quantified using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN; lower limit of detection, 2.6 log copies/mL).

Determination of nucleotide sequences of HBV DNA

DNA was extracted from 100 μL of serum. PCR reactions for detection of the reverse transcriptase (rt) region (nt 130–1161) of HBV DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense; 5′-CTGTGGAAGGCTGGCATTCT-3′) and BGR2 (antisense; 5′-GGCAGGATAGCCGCATTGTG-3′), and PreSBamH1 (sense; 5′-CTTGGGATCCAGAGCTAC AGCATGG-3′) and BR112 (antisense; 5′-TTCCGTCG ACATATCCCATGAAGTTAAGGGA-3′), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification with 94 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense; 5′-GGCCAAGTCTGTACAA CATC-3′) and B12R (antisense; 5′-TGCAGAGGTG AAGCGAAGTG-3′), and B11F and B14R (antisense; 5′-GATCCAGTTGGCAGCACACC-3′), respectively, under the same conditions. The amplified PCR products were used for direct sequencing or cloning methods as previously described [19, 20]. When mutations as a mixed viral population with the wild type sequence for direct sequencing were present, PCR was performed using a cloning method. Sequences of 9–26 independent clones from the sample were determined and analyzed. Measurement of sequences in the rt region was performed at the start of ADV treatment, and on viral breakthrough (VBT) during ADV plus LAM combination therapy. VBT was defined as any increase in serum HBV-DNA by >1 log copies/mL from the nadir or redetection of serum HBV-DNA at levels tenfold the lower limit of detection of the HBV-DNA assay after having an undetectable result. Moreover, sequences for serum HBV DNA level of ≥4 log copies/mL after 1 or 2 years of ADV plus LAM combination therapy were also measured.

Measurement of LAM-, ADV-, ETV- and TDF-resistant variants using ultra-deep sequencing

Ultra-deep sequencing was performed using the Ion Personal Genome Machine (PGM) Sequencer (Life Technologies), as described previously [21]. An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick-translated and PCR-amplified for a total of eight cycles. Subsequently, the library was purified using AM-Pure beads (Beckman Coulter, Brea, CA) and the concentration was determined using the StepOne Plus Real Time PCR (Life Technologies) and Ion Library Quantitation Kit in accordance with the manufacturer's instructions. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with an Ion OneTouch 200 Template Kit v2 (Life Technologies). Enrichment for templated ion spheres particles (ISPs) was performed using the Ion OneTouch Enrichment System (Life Technologies) in accordance with the manufacturer's instructions. Templated ISPs were loaded onto an Ion 314 chip and subsequently sequenced using 130 sequencing cycles in accordance with the Ion PGM 200 Sequencing Kit user guide. Total output read length per run is over 10 M base (0.5 M-tag, 200 base read). The results were analyzed with the CLC Genomics Workbench software (CLCbio, Aarhus, Denmark). A control experiment was included to validate the error rates in ultra-deep sequencing of the viral genome. In this study, amplification products of the second-round PCR were ligated with plasmid and transformed in Escherichia coli in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived rt sequence was determined as the template by the control experiment. Coverage per position for aa180, aa181, aa184, aa194, aa202, aa204, aa233, aa236 and aa250 in the rt region was 63320, 63890, 67737, 49273, 57410, 57211, 40155, 34801 and 42914, respectively. Thus, using the control experiment based on the plasmid encoding rt sequence, amino acid mutations were defined as amino acid substitutions at a ratio of more than 0.25 % of total coverage. This frequency ruled out putative errors caused by the deep sequence platform used in this study.

HBV genotype

The major genotypes of HBV were determined using the enzyme-linked immunosorbent assay (ELISA, Institute of Immunology, Tokyo, Japan) or the PCR-invader assay (BML, Inc, Tokyo, Japan) according to the method described by Usuda et al. [22] or Tadokoro et al. [23].

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 or Fisher's exact test where appropriate. Independent risk factors predicting the achievement of HBeAg seroclearance were studied using stepwise Cox regression analysis. The following 14 potential predictors of HBeAg seroclearance were assessed in this study: age, sex, pretreatment with IFN, severity of liver disease (CH or liver cirrhosis), duration from LAM to ADV, substitution of rtM204, HBV genotype, and levels of aspartate aminotransferase (AST), ALT, bilirubin, albumin, γ-glutamyl transpeptidase, platelets, and HBV DNA. Each was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBeAg seroclearance ($P < 0.10$) were tested in the multivariate Cox proportional hazards model, and hazard ratios and 95 % confidence intervals (CIs) were calculated to assess the relative risk confidence. The above calculations were performed using the Windows IBM SPSS version 19.0.0 software (IBM Corp., Armonk, NY, USA). A Kaplan–Meier estimate was also performed using the SPSS software.

Results

Study population

Clinical and virological profiles of the 406 patients at the start of ADV plus LAM combination therapy are shown in Table 1. At the start of combination therapy, 81 patients (20 %) had cirrhosis and 208 (51 %) were positive for HBeAg. Fourteen (3 %), 25 (6 %), 364 (90 %), 2 (0.5 %), and 1 (0.2 %) patients were infected with HBV genotypes A, B, C, D, and F, respectively. During the clinical course, 48 of 406 patients (12 %) showed an elevation in serum creatinine >1.2 mg/dL, and their ADV dose was accordingly reduced to 10 mg every second day.

Response to ADV plus LAM combination therapy

The ratio of patients with undetectable serum HBV DNA levels (<2.6 log copies/mL) was 63 % (231/367), 72 % (254/352), 75 % (249/331), 79 % (235/297), 82 % (210/256), 80 % (137/171), and 85 % (94/110) at years 1 through 7, respectively (Fig. 1a). Among HBeAg-positive patients at baseline, undetectable rates of serum HBV DNA levels gradually increased from 1 to 7 years (42, 57, 65, 70, 76, 75, 83 % at years 1 through 7, respectively; $n = 208$). In contrast, ratios in HBeAg-negative patients at baseline were >80 % at all points (86, 89, 88, 90, 91, 87, 89 % at years 1 through 7, respectively; $n = 193$). The undetectable rates of serum HBV DNA in HBeAg-negative patients

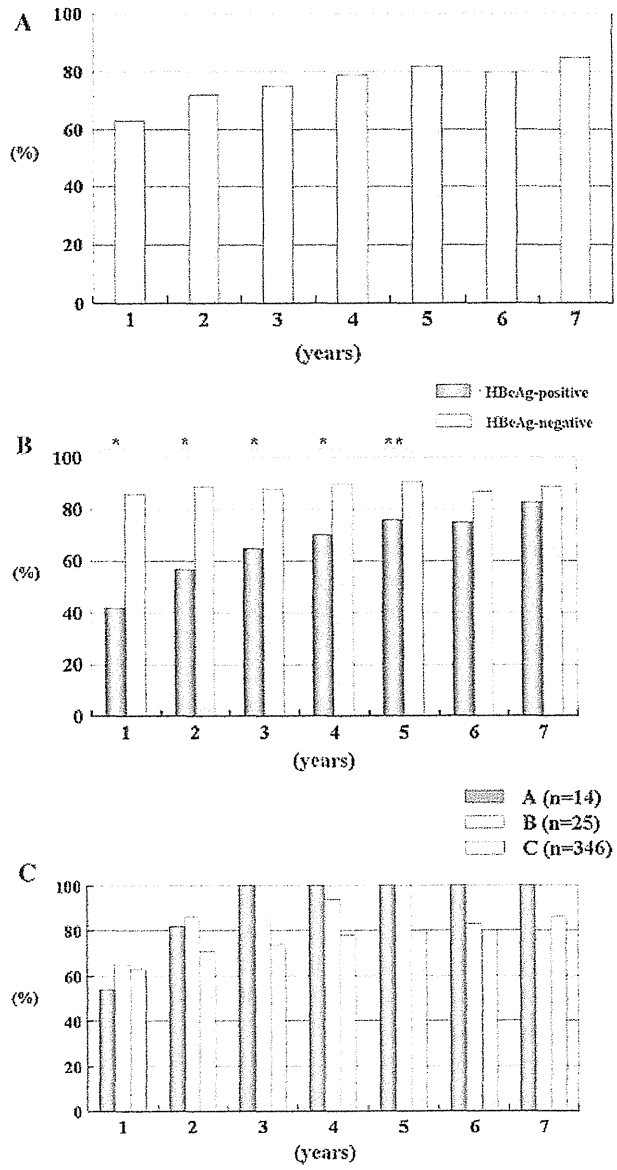

Fig. 1 Undetectable serum HBV DNA levels (<2.6 log copies/mL) in years 1 through 7, respectively. **a** All patients. **b** HBeAg status. A *single asterisk* indicates a statistical significance of $P < 0.0001$ and a *double asterisk* indicates $P = 0.0044$, as determined at the χ^2 test. **c** Genotypes A, B and C

were significantly higher than those in HBeAg-positive patients at years 1 through 5 ($P < 0.0001$ at years 1 through 4, and $P = 0.0044$ at year 5) (Fig. 1b).

By genotype, serum HBV DNA levels were undetectable after 3 years in 100 % of those with genotype A (54, 82, 100, 100, 100, 100, 100 % at years 1 through 7, respectively; $n = 14$), and in >80 % after 2 years in those with genotype B (65, 86, 85, 94, 100, 83, 80 %, at years 1 through 7, respectively; $n = 25$). In contrast, ratios in patients with genotype C gradually increased from 1 to 7 years (63, 71, 74, 78, 80, 80, 86 %, at years 1 through 7, respectively; $n = 364$) (Fig. 1c).

Moreover, the ratio of patients with ALT normalization (≤30 IU/L) was 66 % (250/380), 73 % (262/358), 78 % (255/327), 77 % (226/292), 77 % (194/251), 76 % (125/165), and 77 % (81/105) at years 1 through 7, respectively.

HBeAg clearance

Eighty-four of 208 HBeAg-positive patients (40 %) achieved seroclearance of HBeAg. Cumulative HBeAg seroclearance rates from the commencement date of ADV plus LAM combination therapy were 13 % at 1 year, 24 % at 3 years, 35 % at 5 years, and 52 % at 7 years (Kaplan–Meier method; Supplementary Figure). No patients experienced the reappearance of HBeAg after seroclearance. Six factors found to be associated with the achievement of HBeAg seroclearance in univariate analysis were: AST upper limit of normal (ULN: 30 IU/L)×2<($P = 0.017$), bilirubin 1.1 < mg/dL ($P = 0.020$), ALT ULN×3 <($P = 0.040$), history of IFN therapy ($P = 0.068$), platelets 150<×10^3 μL ($P = 0.074$), and non C genotype ($P = 0.081$). In multivariate analysis, independent factors predicting the achievement of HBeAg seroclearance were history of IFN therapy ($P = 0.009$), AST ($P = 0.016$), bilirubin ($P = 0.030$), and genotype ($P = 0.042$) (Table 2).

HBsAg clearance

Eight of 406 patients (1.9 %) achieved seroclearance of HBsAg (Supplementary Table). All patients were older than 40 years, and all but one was male. Three, two, and three patients were infected with HBV genotypes A, B, C, respectively; two patients were HBeAg-positive at baseline of combination therapy; and five patients had a history of IFN therapy. The duration of HBsAg seroclearance was 2.1–6.8 years.

Genotypic analysis of ADV- and ETV-resistant mutants at baseline of combination therapy and clinical course

Genotypic resistance to LAM, ADV, ETV or TDF was analyzed in baseline samples before the start of ADV plus LAM combination therapy. Substitutions were assessed by direct sequencing or cloning, namely those at rtL180 or rtM204 associated with LAM resistance; rtA181, rtI233, or rtN236 associated with ADV resistance; rtT184, rtS202, or rtM250 associated with ETV resistance; and rtA194 associated TDF resistance. At baseline, substitutions associated with resistance to ADV or ETV were identified in 11 patients (2.7 %) (Table 3). RtA181S/T mutations without substitution at rtM204 were identified in four patients, whereas rtA181T mutation with substitution at rtM204 on the same clones was identified in three patients. RtA181T mutation and rtM204V/I mutation, which existed together on other clones, was identified in two patients. Substitutions related with ETV resistance were identified in the remaining two patients. All but one (Pt. 11) patient was HBeAg-positive and most were younger (<40 years old) and had a high viral load at baseline of LAM therapy. In the remaining 395 patients, rtM204 mutations without substitutions associated with resistance to ADV, ETV or TDF were identified in 358 patients, whereas 37 patients had no substitutions associated with resistance to LAM, ADV, ETV or TDF.

Table 2 Factors associated with HBeAg seroclearance due to ADV plus LAM combination therapy on univariate and multivariate analyses

Parameter	Univariate analysis		Multivariate analysis	
	Hazard ratio (95 % CI)	*P*	Hazard ratio (95 % CI)	*P*
AST (≤UNL×2/UNL×2<)	1.717 (1.102–2.676)	0.017	1.750 (1.112–2.754)	0.016
Bilirubin (≤1.1/1.1<)	1.783 (1.095–2.903)	0.020	1.743 (1.056–2.876)	0.030
ALT (≤UNL×3/UNL×3<)	1.577 (1.008–2.468)	0.040		
History of IFN therapy (−/+)		0.068	1.824 (1.164–2.857)	0.009
Platelets (≤150 × 10^3/150 × 10^3<)		0.074		
Genotype (C/non C)		0.081	2.096 (1.025–4.274)	0.042

HBeAg hepatitis B e antigen, *ADV* adefovir dipivoxil, *LAM* lamivudine, *CI* confidence interval, *AST* aspartate aminotransferase, *UNL* upper limit of normal: 30 IU/L, *ALT* alanine aminotransferase, *IFN* interferon

Table 3 Characteristics of patients with resistance to ADV, ETV or TDF at baseline of ADV plus LAM combination therapy

No.	Baseline of LAM therapy					Baseline of ADV plus LAM combination therapy	
	Age	*Sex*	*Genotype*	*HBeAg*	*HBV DNA level*	*Mutation type (rt region)*	*Duration from start of LAM to emergence of mutation (years)*
1	29	M	C	+	7.6<	A181S	3.3
2	32	M	C	+	7.6<	A181T	1.3
3	23	M	C	+	7.6	A181T	2
4	34	M	C	+	nd	A181T	5
5	35	M	C	+	7.6<	A181T (17/19), L180M + M204V (2/19)	1
6	37	M	C	+	6.5	A181T (7/24), M204I (15/24), L180M + M204V (2/24)	1.3
7	51	M	C	+	7.4	A181T + M204I	1.3
8	38	F	C	+	nd	A181T + M204I (7/13), M204I (6/13)	4
9	33	M	C	+	nd	A181T + M204I (10/21), A181T + M204V(1/21), M204I (10/21)	1.3
10	25	F	D	+	nd	L180M + S202G + M204V	5
11	31	F	C	−	7.6<	L180M + M204V + M250L	6

No. of clones with combined mutations in rt region/total clones are shown in parentheses

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *nd* not done, *rt* reverse transcriptase, *M* male, *F* female

Following ADV plus LAM combination therapy, HBV DNA levels of four patients (Pt. 5, 6, 8, 10) were undetectable (<2.6 log copies/mL) (Fig. 2a), while those of the remaining seven were ≥2.6 log copies/mL. One patient (Pt. 7) achieved HBeAg clearance at 2 weeks, while HBeAg reappeared in a second patient (Pt. 11) at 40 weeks. Ratios of patients with undetectable levels of HBV DNA were 9 % (1/11) at 1 year, 22 % (2/9) at 2 years and 50 % (4/8) at 3 years. Three patients (Pt. 1, 2, 9) received TDF plus LAM or TDF plus ETV therapy after ADV plus LAM combination therapy due to insufficient virological response. Mutations of rtA181T + rtM204I, rtA181T + rtM204V and rtM204I in Pt. 9 changed to rtA181T + rtN236T and rtL180V + rtM204V after 3 years of combination therapy, and HBV DNA level was again thereafter elevated.

Genotypic analysis of ADV- and ETV-resistant mutants during combination therapy and clinical course

Genotypic resistance to ADV, ETV or TDF was analyzed during ADV plus LAM combination therapy in 395 patients without ADV- or ETV-resistant mutants at baseline. During combination therapy, substitutions associated with resistance to ADV or ETV were identified in 12 patients (3 %) (Table 4). All patients were genotype C and had a high viral load (>5.0 log copies/ml) at baseline of combination therapy. Substitutions of rtM204 were identified in all but one patient (Pt. 19) at baseline. RtA181V/S/

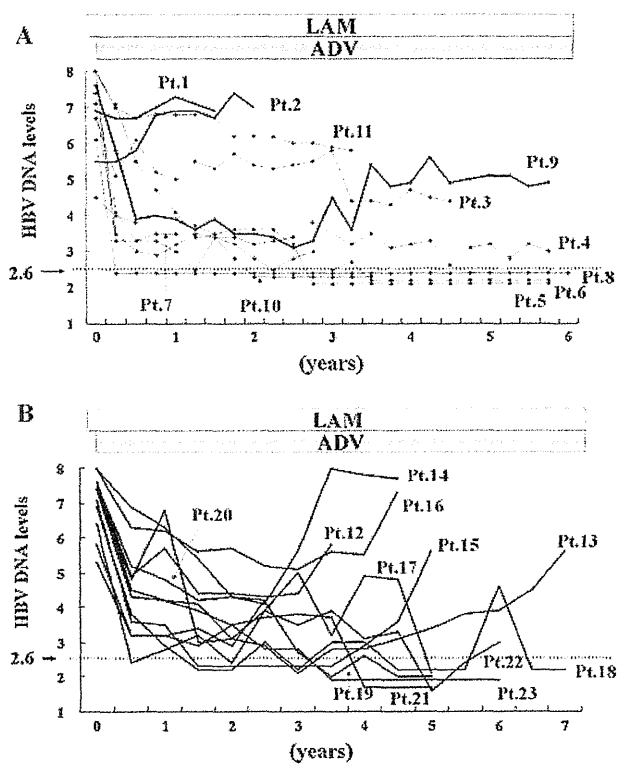

Fig. 2 Clinical course (HBV DNA load) of patients. **a** Patients with resistance associated with ADV or ETV at baseline of ADV plus LAM combination therapy. **b** Patients with resistance associated with ADV or ETV during ADV plus LAM combination therapy

Table 4 Characteristics of patients with emergence of resistance to ADV, ETV or TDF during ADV plus LAM combination therapy

No.	Baseline of ADV plus LAM combination therapy						During ADV plus LAM combination therapy	
	Age	Sex	Genotype	HBeAg	HBV DNA level	rtM204 mutant type	Mutation type(rt)	Duration from start of ADV + LAM to emergence of mutations (years)
12	32	M	C	+	7.6	M204I/V	A181T + N236T (12/16), L180M + N236T (1/16), A181T (1/16), L180M + A181T + M204V (1/16), L180M + M204V + N236T (1/16)	1
13	29	M	C	+	7.6	M204I/V	A181T + M204I + M250L (13/18), L180M + M204V + M250L (2/18), L180M + T184I + M204I + M250L(1/18), L180M + M204I + M250L(1/18), A181T + M204I (1/18)	5
14	58	M	C	+	7.6 <	M204I	L180M + T184I + M204I + M250L(16/26), L180M + T184I + M204I(6/26), A181T(4/26),	3.5
15	49	M	C	+	5.1	M204I/V	A181V + M250L	5
16	46	M	C	+	7.6	M204V	A181T + N236T	3
17	30	F	C	+	7.4	M204I	A181T	0.2
18	40	M	C	+	6.9	M204I	A181S	4
19	40	M	C	+	5.3	M204	A181S	2.3
20	49	M	C	+	7.6	M204V	A181V	0.1
21	63	M	C	−	5.8	M204I	A181T(10/11), A181T + M204I(1/11)	2
22	56	M	C	−	6.4	M204V	A181S	0.6
23	36	M	C	+	7.4	M204I	M180M + A181T(5/9), L180M + A181T + M204I + M250I (3/9), L180M + M204I + M250I(1/9)	1

No. of clones with combined mutations in the rt region/total clones are shown in parentheses

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *rt* reverse transcriptase, *M* male, *F* female

T mutation with or without substitution at rtM204 was identified in all patients, whereas rtT184I or rtM250I/L mutation with or without substitution at rtM204 was identified in 4 patients. Moreover, rtA181T + N236T double mutation related with ADV resistance was identified in two patients (Pt. 12 and 16). Interestingly, substitutions of rtM204 were not detected in five patients (Pt 15, 17, 18, 19, 22) when these ADV- or ETV-related mutations emerged.

Following ADV plus LAM combination therapy, the ratio of patients with undetectable levels of HBV DNA was 0 % (0/12) at 1 year, 25 % (3/12) at 2 years, 27 % (3/11) at 3 years, and 20 % (2/10) at 4 years (Fig. 2b). The HBV DNA levels of five patients (Pt. 12–16) were re-elevated after a decrease, and these patients were then switched to a different treatment (TDF plus LAM or TDF plus ETV in four patients and ETV plus ADV in one). Two of these five patients (Pt. 12 and 16) had rtA181T + rtN236T double mutation-related ADV resistance, while three (Pt. 12–14) had a wide variety of mutations. In contrast, HBV DNA levels of patients who had HBeAg clearance (Pt. 17–19, 23) during ADV plus LAM combination therapy were sustained at ≤5 Log copies/mL after 1 year, and only three patients (Pt. 19, 21, 22) showed sustained levels of ≤2.6 Log copies/mL after 4 years.

Evolution of LAM-, ADV-, ETV- and TDF-resistant variants using ultra-deep sequencing

In 10 of 12 patients with emergent substitutions associated with resistance to ADV or ETV during combination therapy, LAM-, ADV-, ETV- and TDF-resistant variants were analyzed by ultra-deep sequencing at baseline (Table 5). Patients 13 and 20 could not be analyzed due to insufficient stored serum. RtA181T/V mutations were detected in all 7 patients by ultra-deep sequencing at baseline, although 6 of these 7 patients had very low frequency (<1 %) variants. Interestingly, rtA181S mutation in 3 patients could not be

Table 5 Detection of resistance to ADV, ETV or TDF by ultra-deep sequencing at baseline in patients with emergence of resistance during ADV plus LAM combination therapy

No.	Baseline of ADV plus LAM combination therapy (ultra-deep sequencing)									During therapy
	rtL180	rtA181	rtT184	rtA194	rtS202	rtM204	rtI233	rtN236	rtM250	Mutation type(rt)
12	L (50.7 %)	A (96.4 %)	T (99.9 %)	A (99.9 %)	S (99.9 %)	I (59.1 %)	I (99.8 %)	N (99.9 %)	M (99.8 %)	A181T,
	M (49 %)	**T (3.5 %)**				V (34.5 %)				N236T
14	L (81.2 %)	A (99.4 %)	T (99.9 %)	A (99.7 %)	S (99.8 %)	I (99.6 %)	I (99.7 %)	N (99.8 %)	M (99.5 %)	A181T, T184I,
	M (15.6 %)	**T (0.56 %)**							I (0.38 %)	M250L
15	L (75.3 %)	A (97.5 %)	T (99.7 %)	A (99.7 %)	S (99.7 %)	I (70.6 %)	I (99.7 %)	N (99.8 %)	M (99.6 %)	A181V,
	M (24.4 %)	S (1.5 %)				V (27.2 %)				M250L
		V (0.75 %)								
16	M (99.3 %)	A (99.7 %)	T (99.9 %)	A (99.7 %)	S (99.8 %)	V (99.5 %)	I (99.7 %)	N (99.8 %)	M (99.4 %) I (0.51 %)	A181T
	L (0.26 %)	**T (0.27 %)**		T (0.27 %)						
17	L (99.8 %)	A (99.7 %)	T (99.9 %)	A (99.9 %)	S (99.9 %)	I (80.3 %)	I (99.7 %)	N (99.8 %)	M (99.7 %)	A181T
		T (0.25 %)				M (19.5 %)				
18	L (87.9 %)	A (98.7 %)	T (99.9 %)	A (99.4 %)	S (99.5 %)	I (98.2 %)	I (99.7 %)	N (99.8 %)	M (98.9 %)	A181S
	M (11.9 %)	T (1.3 %)		T (0.55 %)		V (1.7 %)			I (0.97 %)	
19	L (99.8 %)	A (98.8 %)	T (99.9 %)	A (99.8 %)	S (99.8 %)	M (99.5 %)	I (99.6 %)	N (99.7 %)	M (99.6 %)	A181S
		T (0.89 %)								
21	L (98.8 %)	A (98.2 %)	T (99.9 %)	A (99.8 %)	S (99.8 %)	I (72.3 %)	I (99.6 %)	N (99.7 %)	M (99.6 %)	A181T
	M (0.96 %)	V (0.99 %)				M (27.0 %)				
		S (0.48 %)				V (0.49 %)				
		T (0.35 %)								
22	M (99.4 %)	A (99.8 %)	T (99.8 %)	A (99.8 %)	S (99.8 %)	V (99.8 %)	I (99.6 %)	N (99.8 %)	M (99.6 %)	A181S
23	L (87.5 %)	A (99.1 %)	T (99.9 %)	A (99.9 %)	S (99.8 %)	I (99.4 %)	I (99.8 %)	N (99.8 %)	M (99.6 %)	A181T, M250I
	M (12.3 %)	**T (0.81 %)**				M (0.48 %)			**I (0.31 %)**	

Bold values indicate emergent substitutions during combination therapy

ADV adefovir dipivoxil, *ETV* entecavir, *TDF* tenofovir disoproxil fumarate, *LAM* lamivudine, *rt* reverse transcriptase

detected at baseline. In contrast, rtT184I, rtN236T or M250I/L mutations were detected in 1 of 4 patients with emergent mutations during combination therapy.

Discussion

Although ADV plus LAM combination therapy is a standard rescue treatment for patients with LAM-refractory HBV, the virological benefits of long-term therapy have not yet been fully assessed. Here, we evaluated the long-term efficacy of ADV plus LAM combination therapy in 406 LAM-refractory patients over a median follow-up period of 5.4 years. We also investigated baseline factors associated with HBeAg clearance and HBsAg clearance. We found long-term combination therapy produced a gradual virological improvement. In particular, virological response was higher in patients who were HBeAg-negative at baseline, and genotype A and B. Toyama et al. [24] recently evaluated the long-term (median 41 months, 158 patients) efficacy of add-on ADV treatment for patients with LAM-resistant HBV and reported a rate of virological response of 90.8 % at 4 years. Inoue et al. [25] reported that HBV-DNA levels were undetectable (<2.6 log copies/mL) on long-term ADV plus LAM combination therapy (median 47 months; 28 patients, including 7 genotype B) in 56, 80, 86, and 92 % of patients at 12, 24, 36, and 48 months, respectively, whereas Aizawa et al. [26] reported undetectable levels on the same long-term regimen (median 46 months, 72 patients) in 61, 74, 81, 84, and 85 % at 12, 24, 36, 48, and 60 months, respectively, a pattern of response that was similar to our present findings. These differences in virologic response among these Japanese studies might have been due to treatment duration, genotype, or number of patients. Nevertheless, all these long-term studies in Japanese showed a gradual increase in virological response rate for 7 years, and that combination therapy with ADV plus LAM was effective for LAM-refractory patients without multidrug-resistant HBV.

The rate of HBeAg clearance at the end of follow-up in our study of 40 % was compatible with previous reports [13, 24]. The strongest predictor of HBeAg clearance on multivariate analysis was IFN history, as in a previous report [24]. Moreover, we recently reported that HBsAg clearance during NA therapy in patients with HBeAg was influenced by previous IFN therapy and HBV genotype [27]. These results suggest that previous IFN therapy might have an immunomodulatory effect on NA therapy. In addition, baseline levels of AST and bilirubin were also significantly associated with HBeAg clearance in this study. Our results agree with those of many clinical studies that have shown baseline transaminase levels to be the strongest predictor of HBeAg seroconversion in response to both IFN [11] and NA therapy [6, 28]. On the other hand, the rate of HBsAg clearance at the end of follow-up in the present study was only 1.9 %. As mentioned above, we reported that HBsAg clearance during NA therapy was influenced by previous IFN therapy and HBV genotype as well as HBsAg level at baseline or by a decrease in HBsAg level within 6 months [27]. That study [27] included patients originally treated with LAM monotherapy or ETV therapy who switched to LAM monotherapy along with ADV plus LAM combination therapy. In this regard, further study to evaluate factors affecting HBsAg clearance in ADV plus LAM combination therapy is necessary.

We previously reported the emergence of ADV-resistant mutations (rtA181T, rtA181S and rtA181T + rtN236T) in 3 of 132 patients at baseline and in 2 during subsequent combination therapy for a period of 2 years [17]. Moriconi et al. [29] reported that rtA181S and rtT184S mutations, either alone or with rtM204 mutation, at baseline in combination therapy in patients with viral breakthrough during LAM monotherapy correlated negatively with virologic response. Moreover, Heo et al. [30] reported that the presence of the rtA181V/T mutation at baseline was associated with a decreased rate of virologic response at 12 months of combination therapy. In the present study, we analyzed more patients with multidrug resistance during combination therapy over a longer clinical course. Substitutions associated with resistance to ADV or ETV were identified at baseline in 11 of 406 patients (2.7 %), most of whom were HBeAg-positive, of younger age, and had a high viral load. Moreover, a virological response during combination therapy was obtained in only four patients. On this basis, substitution of rtA181 without rtM204 mutation might correlate with a poor virological response in combination therapy. In contrast, virological response rate in patients with mutations associated with ETV (Pt. 10 and 11) was 50 %. Inoue et al. [25] detected ETV-resistant mutations of rtT184S and rtS202C during ADV plus LAM combination therapy, and noted that these patients also showed an ADV resistance profile on in vitro analysis. Moreover, a previous report showed that A181S, A181S + M204I, and L180M + T184S + M204V/I mutations were associated with a poor response to ADV plus LAM combination therapy [29]. In light of these results, A181S mutation and A181T without rtM204I/V mutation at baseline might be associated with multidrug resistance.

On the other hand, substitutions associated with resistance to ADV or ETV were identified in 12 of 395 patients (3 %) during combination therapy. Two patients (Pt. 12 and 16) in this group and a patient (Pt. 9) with rtA181T + M204V/I mutations at baseline developed rtA181T + rtN236T double mutation-related ADV resistance. Considering our clinical study, rtA181T + rtN236T

double mutation correlated with a poor virological response. Moreover, a wide variety of mutations (Pt. 12–14) might be correlated with a poor virological response. Inoue et al. reported that 1 of 28 patients developed virologic breakthrough after combination therapy and sequence analysis identified a wide variety of mutations, including L180M + A200V + M204V + N236T, L180M + A200V + M204V, L180M + M204V, L180M + T184S + M204V and L180M + S202C + M204V [25]. The replication capacity of each clone differed [25], and accordingly a wide variety of mutations might be associated with the development of multidrug resistance. Although rtA181S mutation emerged in three patients (Pt. 18, 19, 22), their HBV DNA level was sustained below 5 log copies/mL. This might be explained by the fact that two of these patients (Pt. 18, 19) had HBeAg clearance during combination therapy while the third (Pt. 22) was HBeAg-negative at baseline. In contrast, Lampertico et al. [31] reported that 9 of 145 (6 %) LAM-resistant patients developed rtA181T/V mutation before and during combination therapy for 4 years, but that HBV DNA levels progressively declined to become undetectable in 7 (78 %). In that report, however, rtA181T and rtA181V mutations were detected as a mixed population together with the wild-type sequence rtA181 in all serum samples. In our study, in contrast, rtA181S/T/V mutations were the major population and may accordingly have influenced the poor virologic response. In any case, response to combination therapy may be influenced by amino acid substitutions other than the well-known mutations associated with LAM, ADV, or ETV resistance, and further in vivo and in vitro studies are required.

Moreover, rtA181T/V mutations were detected by ultra-deep sequencing at baseline in 7 of 10 patients with emergent substitutions associated with resistance to ADV or ETV during combination therapy. It was possible that these mutant viruses increased during combination therapy. However, rtA181S, rtT184I or rtN236T or M250L were not detected at baseline. These data indicate that resistant variants of a minor population increased in some cases, whereas de novo resistant variants emerged during combination therapy in others. However, the number of patients analyzed by ultra-deep sequencing in this study was small; and we did not obtain data from patients without emergent substitutions associated with resistance during combination therapy. Further studies should be performed to interpret the significance of the presence of low frequency variants detected by ultra-deep sequencing.

In conclusion, this study shows that long-term ADV plus LAM combination therapy is effective for LAM-refractory patients. A history of IFN therapy, AST, bilirubin, and genotype were important factors in predicting HBeAg seroclearance. However, some patients did not achieve complete viral suppression of HBV DNA level (<2.6 Log copies/mL). We speculate that incomplete suppression might favor further selection of drug-resistant mutants, albeit that the frequency of multidrug resistance in the present study (5.7 %, 23/406) was low. Moreover, the presence of rtA181S mutation at baseline and emergence of rtA181T + rtN236T double mutation or a wide variety of mutations during combination therapy might be associated with a poor virological response. Several recent reports have indicated the effectiveness of TDF for ADV- or ETV-refractory patients [32–34]. Where indicated, HBV DNA and virological analysis should be carefully monitored.

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Conflict of interest The authors declare that they have no conflict of interest.

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